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Original Research

Validation of *In Vitro* Model of Oral Fibrosis Via Genetic and Protein Characterization

Muhammad Shahrukh Khan Sadiq[1,2], Saima Akram Butt[2], Shumaila Usman[3]*, Sana Mirza[4], Anaam Islam[2,5]

1. Department of Oral Pathology, Bahria University Dental College, Bahria University Health Sciences Campus, Karachi, Pakistan. drshahrukhkhan1992@gmail.com
2. Department Oral Pathology, Ziauddin College of Dentistry, Ziauddin University, Karachi, Pakistan. saima.butt@zu.edu.pk
3. College of Molecular Medicine, Ziauddin University, Karachi, Pakistan.
4. Department of Oral Pathology, Institute of Dentistry, Liaquat University of Medical and Health Sciences, Jamshoro, Sindh, Pakistan. sana.mirza@lumhs.edu.pk
5. Department of Oral Pathology, Bolan University of Medical & Health Sciences, Quetta, Pakistan. anaamislam@yahoo.com

***Corresponding Author:** Shumaila Usman, Associate Professor, College of Molecular Medicine, Ziauddin University, Karachi, Sindh, Pakistan. Tel: +92-336-1882779; Email: shumaila.usman@zu.edu.pk

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Abstract.

Background: Oral submucous fibrosis (OSMF) poses a significant health challenge due to its association with restricted mouth opening and an elevated risk of oral cancer. Despite its impact, a lack of well-characterized and validated *in vitro* models has impeded a thorough understanding of the molecular mechanisms driving OSMF progression. ***Objective:*** The primary objective of the present study was to establish and characterize *in vitro* primary cell lines, consisting of fibroblasts, from OSMF biopsy samples. The secondary objectives included assessing the comparative differences in cell viability, population doubling time, gene expression and morphological changes of OSMF cell line fibroblasts, control fibroblasts and OSMF tissue. ***Materials and Methods:*** The study involved the isolation of fibroblasts from tissue affected by OSMF. Following isolation, the fibroblasts were cultured and passaged, with the process continued up to passage 2. Cell viability and population doubling time calculated cell proliferation, while morphological changes were reported. Immunocytochemistry assessed fibroblast markers (vimentin, β-actin and CD90), and qPCR measured gene expressions (*TGFβR-1, COMP, TGM-2, TIMP-1, MMP-7, MMP-9)* across passages, comparing with OSMF tissue and control fibroblasts. **Results:** The *in vitro* OSMF cell line demonstrated successful isolation, subculturing, and sustained fibroblast proliferation. Population doubling time calculations and

morphological observations from passages 0 to 2 provided insights into growth patterns. Immunocytochemistry confirmed fibroblast identity through positive expression of vimentin, β-actin and CD90. qPCR revealed varied expressions of pathological genes across passages, closely resembling OSMF tissue, while distinct differences were noted as compared to control fibroblasts. ***Conclusion:*** The *in vitro* OSMF primary cell line was developed from the patient sample. Validation of this model of oral fibrosis was achieved successfully through genetic and protein characterization, therefore, presenting it as a reliable experimental platform for future investigations into OSMF pathogenesis and potential therapeutic interventions.

Clinical application: This study provides a validated in vitro model for OSMF, offering a foundation to explore disease mechanisms, identify biomarkers, and develop targeted therapies. By closely mimicking the molecular and cellular characteristics of OSMF, the model facilitates testing of potential treatments, understanding fibrosis-to-cancer transitions, and tailoring personalized interventions. It holds potential for advancing precision medicine, improving early detection, and guiding regenerative therapies to restore oral function in affected patients.

Keywords: Cell line; Fibroblasts; Gene Expression; Immunocytochemistry; Oral Submucous Fibrosis.

1. Introduction

Oral submucous fibrosis (OSMF) is referred as a chronic debilitating premalignant condition that affects the oral mucosa that is primarily characterized by inflammation and fibrotic changes in underlying tissues, leading to epithelial atrophy[1,2]. Areca nut consumption serves as the primary risk factor for OSMF, triggering the release of inflammatory mediators that modulate genes and proteins involved in inflammation and cytokine regulation[3]. Predominantly prevalent in south and southeast Asian populations, the global spread of OSMF is due to gradual migration patterns therefore, exacerbating its impact on public health[4]. It is principally classified as an "Oral potentially malignant disorder" (OPMD) by the World Health Organization (WHO), which necessitates urgent research into its effective preventive and therapeutic strategies[5].

Despite advancements, effective treatments for OSMF still remain elusive, as patients are mostly exposed to palliative management[6]. Utilizing *in vitro* oral models of fibrosis can enable researchers to explore the impacts of potential drugs or toxins on cells within a more authentic setting, thereby enhancing predictive efficacy[7]. In this regard, patient-derived primary cell lines can serve as invaluable *in vitro* models for studying the biological processes underlying fibrotic diseases such as OSMF, and also act as essential platforms for screening the efficacy of therapeutic agents[8]. Primary cell lines, obtained directly from living tissue samples, retain many characteristics of the original tissue, providing a more accurate representation of the *in vivo* environment for applications such as drug discovery, toxicity testing, and basic research[9]. Moreover, these cell lines offer several advantages, including ease of growth, cost-effectiveness, and the capability to perform high-throughput analyses of potential treatments[10].

Establishing such an *in vitro* cell line, that can serve as a viable experimental model, from OSMF samples presents challenges owing to their fibrotic nature, complex isolation procedures, and cellular senescence[11]. However, the present study successfully established a novel *in vitro* oral fibrosis model from a biopsy sample obtained from a patient with OSMF condition. Our primary objective was to establish and characterize the cell line of OSMF by isolating fibroblasts from patient samples, aiming to create a physiologically relevant model system for further investigation.

The study involved establishing a primary cell line by isolating fibroblasts from tissue affected by OSMF, which were then cultured and subcultured up to passage 2. Cell viability and Population doubling time (PDT) were calculated to assess cell proliferation, while morphological changes were documented. Immunocytochemistry was performed to evaluate fibroblast markers vimentin, β-actin, and CD90, and quantitative PCR (qPCR) was used to measure gene expressions of *TGFβR-1, COMP, TGM-2, TIMP-1, MMP-7, and MMP-9* across passages. These results were compared with those from OSMF tissue and control fibroblasts to validate the model.

2. Materials and Methods

2.1. Patient Selection

The present study was performed from January to December 2023 in accordance with the ethical principles of the Declaration of Helsinki and approved by the Ethical Review Committee (ERC) of Ziauddin University under reference code 3820521MSOM. The patients with OSMF and healthy participants were enrolled from Dr. Ziauddin Dental Hospital at the Clifton campus, with laboratory work conducted at the Multidisciplinary Research Laboratory at Ziauddin University's Clifton campus, Karachi. A total of 10 participants were included, with 5 OSMF patients and 5 control subjects. OSMF patients were clinically diagnosed, above 18 years of age, and classified as moderate cases according to the Bose and Balan Classification (2007)[12]. Exclusions included those receiving treatment for OSMF, using medications affecting fibroblastic activity, or with comorbidities like diabetes, hypertension and ischemic heart disease. Control subjects were patients above 18 years undergoing third molar impaction surgery, excluding those with habits such as areca nut consumption, tobacco chewing, smoking, or with comorbidities and medications affecting fibroblastic activity.

2.2. Sample Collection and Preparation

Tissue samples were obtained by performing a biopsy procedure of buccal mucosa in the posterior region, during the extraction of the third molar in the control group, while a biopsy of fibrotic areas in the buccal mucosa was collected for the experimental group. To mitigate concerns regarding microbial contamination, participants were instructed to utilize antimicrobial chlorhexidine mouthwash before the biopsy procedure. Of the initial ten samples, only six were successfully processed due to contamination issues. However, only one sample from the OSMF group and one from the control group were ultimately analyzed, as other samples were discarded due to contamination during processing.

2.3. Tissue Processing and Cell Culture

The samples obtained from the dental hospital were transferred to the laboratory in Phosphate Buffered Saline (PBS) (Thermoscientific CAT# 10010023), for cell culture. Upon arrival, tissue samples were initially digested in trypsin 10x for 5-7 minutes. After digestion, the samples were minced into small pieces on a petri dish containing Dulbecco's Modified Eagle's Medium (DMEM) culture media (Thermoscientific CAT# 11965092). Following mincing, the samples were washed in PBS supplemented with antibiotics and fungicides to eliminate any contaminants. Subsequently, the samples were cultured in the medium supplemented with 10% Fetal Bovine Serum (FBS) (Thermoscientific CAT# 10270106) and penicillin-streptomycin (Thermoscientific CAT# 15140122) under sterile conditions to prevent contamination. The cells were maintained in an incubator set at a temperature of 37°C with 5% CO_2 and regularly monitored for growth and viability. Upon reaching 80% confluency, the cells were sub-cultured into new cell culture flasks. The cells were washed twice with PBS and then treated with 0.1% trypsin (Thermoscientific CAT# 15090046) for 3 minutes at 37°C. The trypsinization process was halted by the addition of a complete medium, and the suspended cells were transferred into a falcon tube. They were then centrifuged for 8 minutes, and the resulting cell pellet was seeded into a sterile tissue culture-treated flask. The subculturing was performed till passage 2.

2.4. Cell Viability Assessment

The viability of the cells in each successive passage (P0, P1, P2) was assessed using the trypan blue staining method and a hemocytometer for rapid quantitative culture count estimation. The cells were diluted in a 1:1 ratio of cell suspension and Trypan Blue dye (Thermoscientific CAT# 15250061), then loaded onto the hemocytometer. Viable cell count (cells/mL) was calculated by multiplying the average cell count by the dilution factor and then by 10,000, while the percentage of viable cells was calculated by dividing the number of viable cells by the total cell count and then multiplying by 100.

2.5. Population Doubling Time

In addition to assessing cell viability, PDT was determined in each passage in order to measure cell proliferation rate. To calculate PDT of cells, the formula was employed was $PDT = (t2 - t1) \times \log(2) / \log(N2/N1)$, where t1 and t2 represent the initial and final time points of the cell culture, respectively, and N1 and N2 represent the initial and final cell counts, respectively.

2.6. Morphological Analysis

Morphological analysis of cells at various passages was conducted using a phase contrast microscope, allowing for a comparison between normal fibroblasts and those affected by OSMF. By visually inspecting cellular characteristics, differences between the two cell types were identified and assessed.

2.7. Immunofluorescence Staining

Immunofluorescence staining of cells was performed after passage 2. Cells were seeded using 24-well plates at an estimate of 10,000 cells per well. After attachment, cells were washed

twice with PBS, after removal of DMEM. Fixation with 4% paraformaldehyde was performed and permeabilization with 0.1% Triton X-100 (Thermoscientific CAT# HFH10) in PBS was conducted for 15 minutes at 25°C. After PBS rinses performed additionally, cells were blocked overnight at 4°C and incubated with rabbit polyclonal primary antibodies against β-actin (Thermoscientific CAT# AM4302), vimentin (Thermoscientific CAT# MA5-11883), and CD90 (Thermoscientific CAT# F15-42-1) at a 1:100 dilution for characterization. Subsequently, cells were again treated with PBS and incubated with a donkey anti-rabbit secondary antibody (1:500 dilution) (Thermoscientific CAT# A16035) at room temperature for 60 minutes. Following PBS washes, nuclei were counterstained with DAPI (1:10,000 dilution). Finally, cells were again washed with PBS and an inverted fluorescence microscope was used for their observation.

2.8. Gene Expression Analysis

The gene expression analysis was conducted after passage 2 for OSMF cell line fibroblasts and control fibroblasts. A biopsy sample of OSMF tissue was also obtained for assessing the expression of genes. Prior to RNA isolation, all consumables, including pipettes, glassware, and the benchtop surface, were cleaned with RNase erase spray to minimize RNA degradation. Following trypsinization, the cell pellet formed was, thus, resuspended in Trizol reagent (1 mL) (Thermoscientific CAT# 15596026). Chloroform was added, and after centrifugation, the upper aqueous phase (consisting of RNA) was collected in a sterile tube. RNA precipitation was acquired by adding chilled 100% isopropanol, followed by incubation and centrifugation. The resulting pellet of RNA was rehydrated with 70% ethanol, dried by air, and then resuspended in nuclease-free water and stored at low temperatures of -80°C. RNA concentration was determined by measuring absorbance at 260 nm using a spectrophotometer (Multiskan sky, Thermoscientific). Afterward, the purity of RNA was determined by calculating the absorbance ratio at 260/280 nm. RNA samples with purity levels between 1.8-2.2 were considered suitable for cDNA synthesis. For the purpose of qPCR, 0.4 µL of cDNA was mixed with 4.6 µL of 1X SYBR green Master Mix (Thermoscientific CAT# K0221) in a PCR tube. To complete the mixture, 5 µL of specific diluted primer was added, resulting in a total volume of 10 µL in each tube. The amplification process followed a specific thermal profile: denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds over 40 cycles. Furthermore, CT values were obtained, and the relative fold change was then calculated by employing ΔΔCt method [13]. The genes assessed and their corresponding forward and reverse primer sequences and product sizes are given in Table 1.

Table 1. Genes and Corresponding Forward and Reverse Primer Sequences

Gene	Aversion No.	Forward Primer	Reverse Primer	Product Size
GAPDH	BC025925	CCAGAACATCATCCCTGCCT	CCTGCTTCACCACCTTCTTG	185
TGFβR-1	NM_004612	AGGATGCTCACCTTCCAAGA	GCTGGAGACAGAAAGGATGG	155
COMP	NM_000095.3	CCAGGACGACTTTGATGCAG	TTGTCTGCACGATCTCCCTT	179
TGM-2	AH003011	GGAAAGGAGGGAGAAGGGAC	CAAGTGAGGCATCGTGTCAG	185
TIMP-1	NM_003254	GCTCTGAAAAGGGCTTCCAG	TGTCCGGAAGAAAGATGGGA	167

MMP-7	NM_002423	GGCTTTAAACATGTGGGGCA	GCGTTCATCCTCATCGAAGT	199
MMP-9	NM_004994	CGCTACCACCTCGAACTTTG	TGCCATTCACGTCGTCCTTA	197

Abbreviations: *GAPDH* Glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene); *TGFβR-1* Transforming Growth Factor Beta Receptor 1; *COMP* Cartilage Oligomeric Matrix Protein; *TGM-2* Transglutaminase 2; *TIMP-1* Tissue Inhibitor of Metalloproteinases 1; *MMP-7* Matrix Metalloproteinase 7; *MMP-9* Matrix Metalloproteinase 9

2.9. Statistical Analysis

SPSS version 24 was used for the analysis of the collected data. Shapiro-Wilk test was used to assess the normality of data and an independent t-test was applied to compare cell viability, PDT, and gene expression between OSMF cell line fibroblasts and control fibroblasts at each passage. For gene expression analysis, the same statistical test was used to compare the expression levels of specific genes (*TGFβR-1*, *COMP*, *TGM-2*, *TIMP-1*, *MMP-7*, and *MMP-9*) between OSMF cell line fibroblasts and control fibroblasts, as well as between OSMF tissue and OSMF cell line fibroblasts. The Levene's test for equality of variances was performed to verify the assumption of homogeneity of variances. Data are presented as means ± standard deviation (SD), and a p -value of < 0.05 was considered statistically significant.

3. Results

3.1. Cell Viability Assay

The trypan blue exclusion method found no significant difference between control and OSMF cell line fibroblasts across passages 0, 1 and 2. However, OSMF cell line fibroblasts showed higher cell viability due to increased proliferation rates. This finding indicates the inherent capacity of fibroblasts for increased proliferation thereby, contributing to their higher viability (Figure 1).

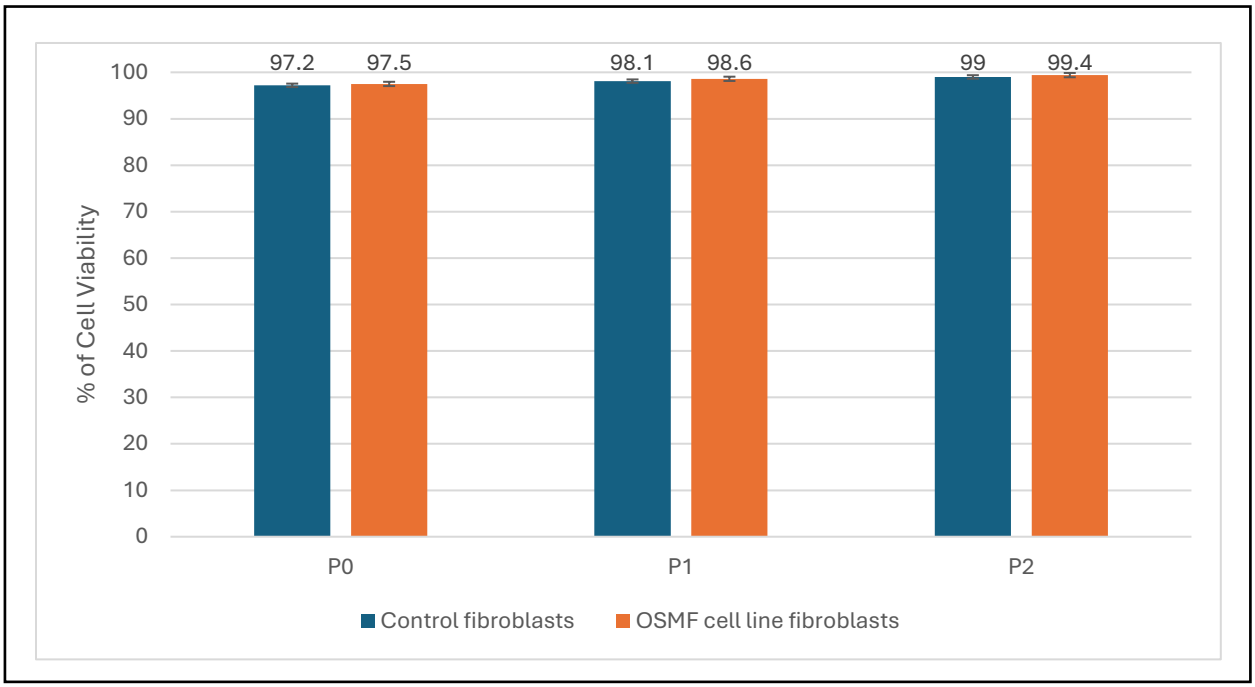

Figure 1. Comparison of cell viability between control and OSMF cell line fibroblasts in passages 0, 1 and 2. No significant difference between control and OSMF cell line fibroblasts was recorded ($P < 0.05$ was considered statistically significant).

3.2. Population Doubling Time

Both OSMF and control fibroblasts displayed a consistent increase in cell count across passages, indicating active proliferation. OSMF cell line fibroblasts exhibited a significantly shorter PDT compared to control fibroblasts across all passages. This suggests that OSMF cell line fibroblasts have a higher proliferation rate and thus a faster rate of population doubling. The shorter PDT observed in OSMF cell line fibroblasts indicates an enhanced proliferative capacity compared to control fibroblasts. The PDT values of OSMF and control fibroblasts for different passages in mentioned in Table 2.

Table 2. Population Doubling Time for OSMF cell line fibroblasts and control fibroblasts

Passage	PDT (hours) for OSMF cell line fibroblasts	PDT (hours) for control fibroblasts	*P*-value
P0	5.77	12.67	0.004*
P1	7.55	13.53	0.002*
P2	8.48	14.39	0.003*
* Significant value ($P < 0.05$)			

3.3. Morphological Analysis

In P0, control fibroblasts exhibited a characteristic spindle-shaped morphology, featuring long, slender bodies and elongated nuclei. OSMF cell line fibroblasts also primarily displayed a spindle shape, but some took on an epithelioid appearance. Advancing to P1, normal oral fibroblasts retained their spindle-shaped form and developed multiple extensions. In contrast, OSMF cell line fibroblasts formed aggregates comprising a mixture of spindle, epithelioid, and stellate-shaped cells. At P2, OSMF cell line fibroblasts predominantly consisted of epithelioid and large stellate-shaped cells, with fewer spindle-shaped cells. Normal fibroblasts at this stage maintained their spindle-shaped appearance with dense, elongated projections. OSMF cell line fibroblasts also tended to form densely packed cellular aggregates, potentially contributing to the development of fibrous bands. As the OSMF passages progressed, the fibroblast morphology became increasingly abnormal, with some taking on an elongated, spindly appearance, while others became more rounded or stellated (Figure 2).

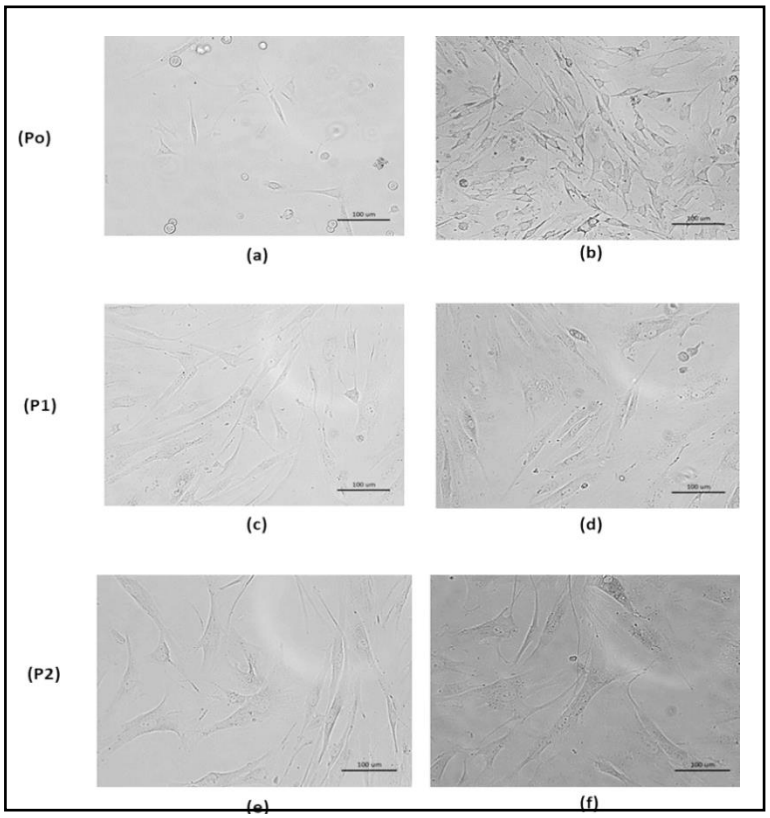

Figure 2. The morphology of OSMF and control fibroblasts was examined at different passages (P0, P1, and P2). In the top panel, control cells are depicted, while OSMF cells are shown in the bottom panel. At P0, both OSMF (a) and control cells (b) display a spindle-shaped morphology with similar size and shape. This morphology persists at P1 (c) and (d). However, at P2, control cells (e) maintain their spindle shape with uniform size and increased density, while OSMF cells (f) exhibit larger and more irregular shapes. However, at P2, control cells (e) maintain their spindle shape with uniform size and increased density, while OSMF cells (f) exhibit larger and more irregular shapes.

3.4. Immunocytochemistry Analysis

Immunocytochemistry analysis confirmed the absence of mycoplasma contamination in both OSMF and control fibroblasts, as indicated by the absence of blue staining in DAPI-stained cytoplasm. Positive staining for the fibroblast-specific markers, vimentin, β-actin and CD90 were observed in both OSMF and control cells using their respective antibodies. The presence of red staining in both cell types affirmed the expression of vimentin, β-actin and CD90, emphasizing their similarity as fibroblasts. (Figure 3).

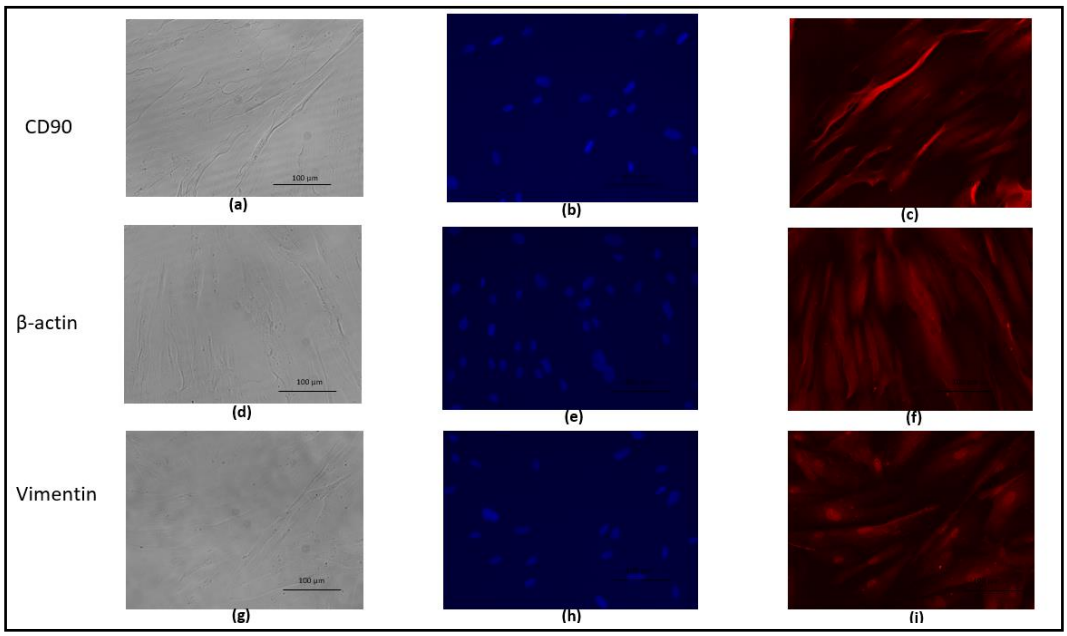

Figure 3. Immunocytochemistry results for CD90, β-actin and vimentin expression in OSMF cell line fibroblasts. OSMF cell line fibroblasts show positive CD90, β-actin and vimentin expression (c) (f) (i), alongside DAPI-stained nuclei (b) (e) (h), as observed in the 40X magnified fluorescence microscopy images.

3.5. Gene Expression Analysis

According to the results, the gene expression profiles of OSMF cell line fibroblasts closely resembled those of OSMF tissue, while showing significant differences from control fibroblasts. Among the examined genes, *TGFβR-1*, *COMP*, *TGM-2*, and *TIMP-1* were significantly upregulated, while *MMP-7* and *MMP-9* were significantly downregulated in OSMF cell line fibroblasts as compared to control fibroblasts (Figure 4). While no significant differences among the genes were found between OSMF tissue and OSMF cell line fibroblasts (Figure 5).

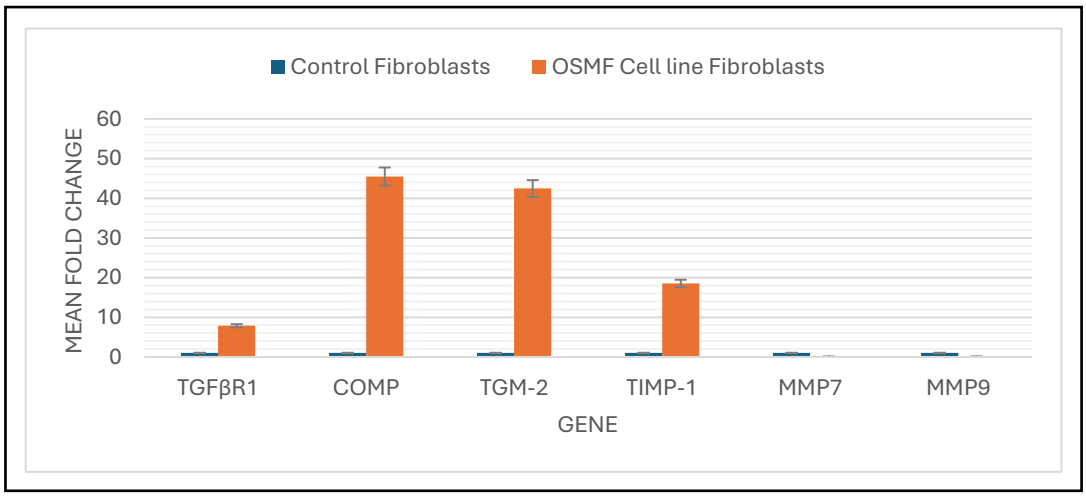

Figure 4. Significant differences between mean fold change of control fibroblasts and OSMF cell line fibroblasts genes were reported. *TGFβR-1*, *COMP*, *TGM-2*, and *TIMP-1* were significantly upregulated, while *MMP-7* and *MMP-9* were significantly downregulated in OSMF cell line fibroblasts as compared to controls. The gene expression levels were determined by the ΔΔCt method relative to the control group (*P* values < 0.05 considered statistically significant).

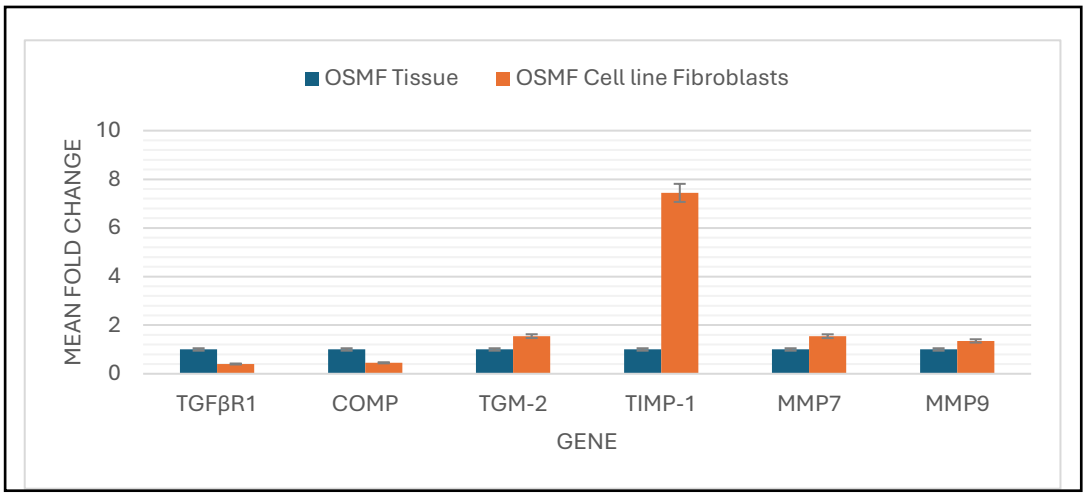

Figure 5. No significant differences between mean fold change of OSMF cell line fibroblasts and OSMF tissue in genes were reported. The gene expression levels were assessed by the ΔΔCt method relative to the control group (*p* values < 0.05 considered statistically significant).

4. Discussion

Establishing *in vitro* models of oral fibrosis by isolating fibroblasts from OSMF tissue presents challenges due to the fibrotic nature of the tissue, intricate isolation procedures, and issues related to cellular senescence[11]. Standard protocols for isolating and establishing primary cell cultures of gingival keratinocytes, gingival fibroblasts and periodontal ligament fibroblasts have been well-documented in the scientific literature[14]. However, there is a scarcity of research specifically focused on primary cultures of fibroblasts derived from patients with OSMF. Limited researches are found in the literature that focus on the morphology and differentiation of *in vitro* fibroblasts by examining primary cell cultures of normal oral fibroblasts as well as fibroblasts obtained from individuals who chew areca nut. In 2011, primary cultures of fibroblasts were successfully established by utilizing the collagenase disaggregation method, and additionally, phenotypic characteristics and growth patterns of these were assessed extensively[15]. Moreover, Banerjee et al. noted morphological variations in OSMF-derived fibroblasts, with arecoline exhibiting dual effects on proliferation and cytotoxicity[16]. Adtani et al. created a synthetic oral fibrosis model by inducing arecoline in healthy fibroblasts, elucidating collagen production dynamics[17]. Our study achieved fibroblast isolation from OSMF tissue, followed by culture and subculture up to passage 2. Assessments included cell viability, PDT, morphological observations, immunocytochemistry for vimentin, β-actin, CD90 and qPCR analysis for key gene expressions (*TGFβR-1*, *COMP*, *TGM-2*, *TIMP-1*, *MMP-7*, *and MMP-9)* across passages and their comparison to OSMF tissue and control fibroblasts, validating our findings through comprehensive genetic and protein characterization. The similarity in gene expression between OSMF cell line fibroblasts and

OSMF tissue supports the reliability of the OSMF fibroblast cell line as a model that closely mirrors OSMF tissue.

PDT serves as a vital indicator of cell proliferation capacity, offering insights into cellular dynamics and pathology. Van Wyk et al. reported no significant difference in growth rates between normal and OSMF fibroblasts, while Ma et al. reported shorter PDT in OSMF fibroblasts compared to normal controls[18,19]. Conversely, in 2011, PDT of OSMF was observed to be three times longer in cell lines from affected individuals compared to controls, as demonstrated by Mathew, Skariah, and Ranganathan[15]. However, our current findings of shorter population PDT in OSMF cell line fibroblasts compared to control fibroblasts indicate that OSMF fibroblasts have a greater tendency for proliferation. This rapid division of OSMF fibroblasts as compared to normal fibroblasts, resultantly leads to their higher cell viability. Nonetheless, our results align with Ma et al. who also observed shorter PDT in OSMF cell line fibroblasts, providing impetus to increased proliferative activity in the cells.

Previous literature has investigated the morphological attributes of both control and OSMF fibroblasts. Research studies by de Waal et al[20] and Banerjee et al[16] highlighted a notable increase in stellate-shaped cells in OSMF compared to control samples, signifying distinct phenotypic alterations in diseased fibroblasts. Our investigation further supports these findings, revealing a dynamic shift in the morphology of OSMF cell lines over successive passages. Initially, spindle-shaped cells predominated, associated with proliferation and migration, while later passages showed an increase in stellate-shaped cells, implicated in collagen synthesis and tissue remodeling. These observations suggest a potential evolution in fibroblast population and phenotype during OSMF progression.

Characterization of primary cell lines is essential for comprehending their biology and potential applications in both research and therapy. Immunocytochemistry, a potent technique for protein localization, was utilized in our study to evaluate vimentin, β-actin and CD90 expression, pivotal markers for mesenchymal cells such as fibroblasts. Fibroblast-specific markers aid in the isolation of these cells from heterogeneous populations, facilitating the investigation of their roles in tissue remodeling and disease progression[21]. Vimentin, an intermediate filament protein prominently expressed in fibroblasts, plays a significant function in tissue repair, cell structure and migration[22,23]. Sawant et al. demonstrated elevated vimentin expression in OSMF fibrotic tissues, correlating with histopathological severity, indicating its involvement in fibrosis progression[24]. CD90, also known as Thy-1, is a glycoprotein present on various cell types, including fibroblasts and stem cells, involved in cell-cell interactions and signal transduction, and is frequently used as a marker for mesenchymal stem cells and fibroblasts; it also performs an essential role in cancer by regulating cell proliferation, metastasis, and angiogenesis[25]. Our immunocytochemistry analysis successfully confirmed the presence of vimentin, β-actin and CD90 in both OSMF and control fibroblasts therefore, validating their fibroblastic identity.

Transforming growth factor-beta (TGFβ) is a multifunctional cytokine that plays a variety of roles in numerous cellular processes that include extracellular matrix (ECM) production, cell growth, differentiation and migration[26]. Prior studies have demonstrated that TGFβ-1 serves as a potent fibrotic mediator in OSMF[27]. Rai et al. explored the molecular mechanisms underlying OSMF by evaluating TGFβ gene expression in OSMF and juxtaposed it to the values of normal tissues. The results revealed a significant upregulation of TGF-β isoforms in OSMF tissues, particularly TGFβ-1, alongside elevated levels of TGFβ receptors (TGFβR-1 and TGβR-2)[28]. In alignment with these investigations, our study also identified significantly elevated expression of the TGFβR-1 gene in OSMF cell line fibroblasts and OSMF tissue compared to control fibroblasts, thus reinforcing the pivotal role of TGFβ signaling in driving the pathogenesis OSMF.

Cartilage oligomeric matrix protein (COMP) is a crucial ECM protein involved in preserving cartilage integrity through interactions with various matrix proteins, including collagen types I, II, and IX, as well as fibronectin. Furthermore, COMP demonstrates increased expression in fibrotic scar regions and lesions associated with systemic sclerosis on the skin, suggesting potential involvement in vascular wall remodeling[29-31]. In a study by Li et al. microarray analysis was conducted to explore differential changes in mRNA of 14,500 genes across OSMF and normal buccal mucosa samples. Among the validated candidate genes, COMP emerged as one of the top up-regulated genes in OSMF[32]. Our study similarly observed significantly elevated expression of COMP in OSMF cell line fibroblasts compared to control fibroblasts, that was similar to the previous findings.

Transglutaminase-2 (TGM-2) is crucial for ECM regulation by facilitating protein cross-linking, essential for tissue integrity and function. Its role in fibrotic conditions like liver fibrosis, pulmonary fibrosis, and OSMF stresses upon its importance in ECM remodeling[33]. In OSMF, elevated TGM-2 levels, driven by arecoline-induced reactive oxygen species (ROS), promote ECM modifications and fibrosis progression. Thangjam et al. demonstrated that arecoline upregulates TGM-2 in fibroblasts, contributing to ECM stabilization[34]. In another study conducted by Shinn-Lee et al. reported significantly increased TGM-2 expression in OSMF tissue from areca quid users, with OSMF-derived fibroblasts showing higher TGM-2 levels than normal samples. Their study also concluded that arecoline induces dose-dependent ROS production and TGM-2 expression in normal buccal mucosa fibroblasts[35]. Our results revealed significantly elevated TGM-2 levels in OSMF cell line fibroblasts compared to controls, with no significant difference between TGM-2 levels in OSMF cell line fibroblasts and OSMF tissue. The consistent elevation of TGM-2 in both OSMF cell line fibroblasts and tissue suggests a localized and potentially paracrine-driven upregulation of TGM-2 within the OSMF microenvironment.

Matrix metalloproteinases (MMPs) are essential for connective tissue turnover, balanced by tissue inhibitors of metalloproteinases (TIMPs) like TIMP-1 and TIMP-2. This balance is vital for normal tissue remodeling and wound healing, as TIMPs inhibit MMPs by binding to their active sites, preventing excessive collagen degradation[36]. Imbalances in MMP and TIMP activities are linked to pathological conditions, including OSMF[37]. In a study by Chang et al. TIMP activity was

examined in cells cultured from OSF and normal buccal mucosa, revealing higher TIMP-1 expression in OSMF specimens[38]. Similarly, Pitiyage et al. reported significant TIMP-1 and TIMP-2 levels in OSMF fibroblasts compared to controls[39-41]. Our study also found a significant increase in TIMP-1 levels in OSMF cell line fibroblasts, alongside a notable decrease in MMP-7 and MMP-9 levels compared to control fibroblasts. These findings confirm that elevated TIMP-1 levels inhibit MMP activity, leading to decreased MMP-7 and MMP-9, and contribute to the excessive ECM accumulation characteristic of OSMF. The apparent increase in TIMP-1 expression in Figure 5 reflects a trend but does not reach statistical significance when compared to OSMF tissue. This difference may be attributed to variability in sample handling or intrinsic differences between *in vitro* and *in vivo* conditions. The 7-fold increase observed in the OSMF cell line compared to OSMF tissue highlights potential upregulation during in vitro adaptation, warranting further exploration into environmental or epigenetic influences on gene expression.

The key limitations of the present study include the exclusion of mild and severe OSMF cases, as well as the challenges posed by microbial contamination during sample processing. Addressing these issues in future studies would enhance the generalizability and reliability of findings. Despite these constraints, the establishment of a novel OSMF cell line provides a critical tool for studying pathogenesis and exploring therapeutic interventions.

5. Conclusion

In conclusion, our study significantly advances the understanding of OSMF by establishing a novel *in vitro* cell line model of oral fibrosis from the OSMF sample. Overcoming the challenges of microbial contamination and processing during its establishment, we provide a valuable tool for researching OSMF pathogenesis. The morphological and molecular characterization of OSMF fibroblasts, coupled with comparisons to existing literature, findings offer promising insights for developing targeted therapies to prevent OSMF progression to oral cancer.

Abbreviations

OSMF	Oral Submucous Fibrosis
OPMD	Oral Potentially Malignant Disorder
WHO	World Health Organization
PDT	Population Doubling Time
ECM	Extracellular Matrix
qPCR	Quantitative Polymerase Chain Reaction
TGFβR-1	Transforming Growth Factor Beta Receptor-1
COMP	Cartilage Oligomeric Matrix Protein
TGM-2	Tissue Transglutaminase-2

TIMP-1	Tissue Inhibitor of Metalloproteinase-1
MMP-7	Matrix Metalloproteinase-7
MMP-9	Matrix Metalloproteinase-9
ERC	Ethical Review Committee
PBS	Phosphate-Buffered Saline
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
CD90	Cluster of Differentiation 90
ROS	Reactive Oxygen Species
SD	Standard Deviation

Declarations:

Supplementary Materials: Not applicable.

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