Activation of TGF-β Pathway by Areca Nut Constituents: A Possible Cause of Oral Submucous Fibrosis

Imran Khan, Neeraj Kumar, Ila Pant, Sivakrishna Narra, Paturu Kondaiah*

Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, India

Abstract

Oral submucous fibrosis (OSF) is a chronic inflammatory disease characterized by the accumulation of excess collagen, and areca nut chewing has been proposed as an important etiological factor for disease manifestation. Activation of transforming growth factor-β signaling has been postulated as the main causative event for increased collagen production in OSF. Oral epithelium plays important roles in OSF, and arecoline has been shown to induce TGF-β in epithelial cells. In an attempt to understand the role of areca nut constituents in the manifestation of OSF, we studied the global gene expression profile in epithelial cells (HaCaT) following treatment with areca nut water extract or TGF-β. Interestingly, 64% of the differentially regulated genes by areca nut water extract matches with the TGF-β induced gene expression profile. Out of these, expression of 57% of genes was compromised in the presence of ALK5 (TβRI) inhibitor and 7% were independently induced by areca nut, highlighting the importance of TGF-β in areca nut actions. Areca nut water extract treatment induced p-SMAD2 and TGF-β downstream targets in HaCaT cells but not in human gingival fibroblast cells (hGF), suggesting epithelial cells could be the source of TGF-β in promoting OSF. Water extract of areca nut consists of polyphenols and alkaldoids. Both polyphenol and alkaloid fractions of areca nut were able to induce TGF-β signaling and its downstream targets. Also, SMAD-2 was phosphorylated following treatment of HaCaT cells by Catechin, Tannin and alkaloids namely Arecoline, Aracaidine and Guvacine. Moreover, both polyphenols and alkaloids induced TGF-β2 and THBS1 (activator of latent TGF-β) in HaCaT cells suggesting areca nut mediated activation of p-SMAD2 involves up-regulation and activation of TGF-β. These data suggest a major causative role for TGF-β that is induced by areca nut in OSF progression.

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* E-mail: paturu@mrdg.iisc.ernet.in

Introduction

Oral submucous fibrosis (OSF) is a chronic inflammatory disease characterized by epithelial atrophy and fibrosis in submucosa of the oral tissues that can cause difficulty in chewing, swallowing, speaking, and mouth opening [1]. Habit of chewing betel nut (Areca catechu) has been proposed to be the most important etiological factor in the development of OSF which is also supported by the development of an in vivo mouse model with areca nut water extract [2,3]. Histopathological findings indicate imbalance between synthesis and degradation of extracellular matrix (ECM), mainly of collagen in the oral sub-mucosa leading to OSF [4]. Synthesis of collagen is governed by the balance of pro and anti-fibrogenic cytokines such as transforming growth factor-β (TGF-β), Endothelin-1, Connective tissue growth factor (CTGF) etc and Bone morphogenetic protein 4 & 7 (BMP4, 7) respectively [5]. The imbalance leading to over-production of pro-fibrogenic cytokines are known to be associated with fibrosis of different organs [6]. Pro-fibrogenic cytokines become key mediators of fibrosis by differentiating fibroblasts to myofibroblast phenotype in connective tissue disorders [7]. In an earlier report, TGF-β was shown to be up-regulated in OSF tissues [8] and its activation has been shown by the nuclear localization of p-SMAD2 in OSF tissues compared to normals [9,10]. This activation of TGF-β signaling in OSF tissues could be due to up-regulation of ligand (TGF-β1) and both its activators, αvβ6 integrin and THBS-1. In addition to matrix synthesis, proteases and matrix cross-linking enzymes play important roles in severity of OSF. Alteration in collagen cross-linking makes it resistant to degradation, leading to fibrosis. There are two major collagen cross-linking enzymes proposed in OSF namely; Lysyl oxidase and Transglutaminase 2 [11,12]. Lysyl oxidase catalyzes formation of aldehydes from lysine residues in collagen and elastin precursors while Transglutaminase-2 (TGM-2) catalyzes transamidating acyltransferase reaction leading to matrix stabilization. These crosslinking enzymes are also known to be affected by the pro-fibrotic cytokines like TGF-β, highlighting the probable role of pro-fibrogenic cytokines in OSF [13,14]. Taken together, these findings suggest that the TGF-β pathway could possibly play an important role in OSF development. Since betel quid chewing habit has been proposed to be the most important etiological factor in OSF pathogenesis, several studies were directed towards establishing a role for arecoline, the principal alkaloid present in betel quid, in OSF pathogenesis. Towards this, there have been reports suggesting regulation of TGF-β and its activation by arecoline in epithelial cells [9,10]. TGF-β activation by arecoline in oral keratinocytes was shown to
Table 1. List of primer sequences used for RT-PCR.

| Sl.No | Genes | F 5'-3' sequence | R 5'-3' sequence | Description |
|-------|-------|------------------|------------------|-------------|
| 1     | TGFβ2 | AGTGCTGAACACGGAT | GTACAAATGTCAGCAGG | 218 bp, 55°C |
| 2     | TGM2  | TGACCTCGGCAAAGACAAAG | CCATGACCAGAACCAGAAGCCT | 241 bp, 50°C |
| 3     | THBS1 | CCGGGCTGAAGTGACTAGCTA | TGCACTTGCGTTCTGTGT | 317 bp, 59°C |
| 4     | TGBF8 | TGTTGCTGAAGGCTGTTT | CGGCCTGTTGAAAGAAGTCA | 313 bp, 50°C |
| 5     | BMP7  | AGGCTGGTTAAGAGCAGGAG | AGGTAGCCGGTGGCTGTC | 268 bp, 59.5°C |
| 6     | TMEPAI | TTCATCGCTCTGGTTTGGG | GCACAAAGCCATGAAATC | 228 bp, 58°C |
| 7     | RPL35A | GAACTAAGGGGACACACAG | CAAATGGCCCTTCAGGAAAGA | 236 bp, 58°C |
| 8     | COL1A1 | TCCCCAGCCACAAAGGTCTA | TTTCCACAGGTTCGGTCA | 201 bp, 58°C |
| 9     | COL2A1 | ACCCTGAGTGGAAAGTGGGA | CCACATTGATGTTCTCC | 199 bp, 58°C |
| 10    | COL1A3 | TTGACCTAAACCCAGGATGC | GGAAATCCAGATTCGGCTA | 201 bp, 58°C |
| 11    | α-SMA | CAGCCAAGCAGCTGTCAGG | CAATGGATGGGAAAACAGC | 150 bp, 59.5°C |

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Figure 1. Hierarchical clustering of TGF-β and Areca nut induced genes. A] Hierarchical clustering of commonly regulated genes in HaCaT cells following treatments with areca nut water extract (5H 5 mg/ml) or TGF-β (5 ng/ml) (P<0.05 and ≥ 1.5 fold). B] Hierarchical cluster of genes that are commonly regulated by areca nut or TGF-β but compromised in presence of ALK5 inhibitor. Red, green and black colours in A and B represent up, down or un-regulated genes respectively. The vertical axis represents genes and horizontal axis represents treatments. C] i and ii, Venn diagram representation of the genes from A and B respectively. doi:10.1371/journal.pone.0051806.g001
be through αvβ6 integrin, suggesting an important role for TGF-β in OSF pathogenesis [9]. However, arecoline is approximately 0.2% in areca nut compared to other compounds such as polyphenols, which are approximately 11–17.8% in areca nut [15]. Hence it is possible that in addition to arecoline, other constituents of areca nut extracts may play important roles in OSF pathogenesis. Therefore using a microarray approach, genes differentially regulated by areca nut extract were identified. Interestingly majority of the differentially regulated genes by areca nut water extract were similar to TGF-β regulated genes. Further, the genes regulated by areca extract were also dependent on TGF-β signaling. We also demonstrate that polyphenols and alkaloids in areca nut were able to induce TGF-β signaling by up regulating TGF-β2 and its activator THBS1. Since polyphenols represent a much higher percentage compared to alkaloids in areca nut, these along with other alkaloids could be major etiological factors of OSF pathogenesis involving TGF-β.

**Materials and Methods**

**Cell lines and treatments**

Primary human gingival fibroblast (hGF) cells were derived from biopsies of Gingival tissues [16] and human keratinocytes (HaCaT) [17] were maintained in DMEM (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Certified grade, Invitrogen corporation, USA. Heat inactivated for HaCaT cells), 100 units/mL penicillin and 100 µg/mL streptomycin (Invitrogen Life Sciences, USA) at 37°C in a humidified chamber with 5% CO2. Human foreskin primary fibroblast cells (FF) (a kind gift by Prof. K. Satyamoorthy, Manipal University, Manipal) were cultured similar to hGF cells as described above. Human Foreskin Keratinocytes (a kind gift by Prof. Annapoorni Rangarajan, IISc) were maintained in Serum-free keratinocyte Medium (Keratinocyte-SFM) supplemented with Bovine Pituitary Extract (BPE 25 µg/ml) and rEGF (Recombinant Epidermal Growth Factor 0.1–0.2 ng/ml). For treatments, cells were serum starved (0.2% serum for hGF and FF cells) for 24 hr and treated with areca nut extracts.

**Figure 2. Areca nut induces TGF-β signaling in epithelial cells.** A & B] Areca nut induced genes by Western blot and by qRT-PCR. HaCaT cells were serum starved for 24 hours and treated with either areca nut water (H) or ethanol (Et) extracts 2.5, 5 µg/ml (2.5H/5H & 2.5Et/5Et) in serum free medium for 48 hours. A) Western blot of HaCaT cell lysates showing induction of p-SMAD2 and its downstream target TGM2. B) qRT-PCR analysis showing expression of TGF-β downstream target genes TGM2, TMEPAI, THBS1 and TGFBI. UN-Untreated, 2.5H & 5H— 2.5 and 5 µg/ml Areca nut water extract, 2.5 Et & 5Et— 2.5 and 5 µg/ml. T-TGF-β (** = P<0.0001 compared to untreated). C & D) ALK5 inhibitor (SB 431542) reverses the Areca nut induced expression of genes in HaCaT cells. HaCaT cells were treated with areca nut water (H) and ethanol (Et) extracts (5H— 5 µg/ml areca nut water extract and 5Et— 5 µg/ml areca nut ethanol extract) in the presence or absence of ALK5 (TβRI) inhibitor. Western blot of p-SMAD2 and TGM2 (C). qRT-PCR of TGM2, TMEPAI, THBS1 and TGFBI (D). Untreated, 5H— Areca nut water extract (5 µg/ml), 5Et— Areca nut ethanol extract (5 µg/ml), ALK5— TβRI inhibitor, 5H+ALK5 inhibtor, 5Et+ALK5 inhibitor (** = P<0.0001 compared to untreated).

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Table 2. Pathways differentially expressed upon areca nut treatment on HaCaT cells as revealed by KEGG pathway analysis.

| Pathways                               | No of Genes | Pathways                               | No of Genes |
|-----------------------------------------|-------------|-----------------------------------------|-------------|
| hsa01100 Metabolic pathways             | 55          | hsa04142 Lysosome                       | 13          |
| hsa05200 Pathways in cancer             | 34          | hsa04010 MAPK signaling pathway         | 13          |
| hsa05166 HTLV-I infection               | 25          | hsa04062 Chemokine signaling pathway    | 13          |
| hsa04110 Cell cycle                     | 24          | hsa05152 Tuberculosis                   | 13          |
| hsa05034 Alcoholism                     | 22          | hsa05169 Epstein-Barr virus infection   | 13          |
| hsa05322 Systemic lupus erythematosus   | 21          | hsa04115 p53 signaling pathway          | 13          |
| hsa05202 Transcriptional misregulation  | 19          | hsa05145 Toxoplasmosis                 | 12          |
| hsa04060 Cytokine-cytokine receptor     | 18          | hsa04141 Protein processing in endoplasmic reticulum | 12          |
| interaction                             |             |                                         |             |
| hsa04114 Oocyte meiosis                 | 18          | hsa04722 Neurotrophin signaling pathway | 12          |
| hsa04510 Focal adhesion                 | 18          | hsa04726 Serotonegic synapse            | 11          |
|                                         |             |                                         |             |
| hsa04144 Endocytosis                    | 16          | hsa04120 Ubiquitin mediated proteolysis | 11          |
| hsa05164 Influenza A                    | 16          | hsa04145 Phagosome                     | 11          |
|                                         |             |                                         |             |
| hsa04810 Regulation of actin cytoskeleton| 14          | hsa05146 Amoebiasis                    | 11          |
| hsa04310 Wnt signaling pathway          | 13          | hsa04912 GnRH signaling pathway         | 10          |

RNA extraction, semi-quantitative and real-time RT-PCR

Total RNA was extracted from HaCaT and hGF cells using TRI-reagent (Sigma-Aldrich, St. Louis, USA) according to the manufacturer’s protocol. Two micrograms of RNA was reverse transcribed using a cDNA synthesis kit (Applied Biosystems, USA) and 1/100th of the reaction product was used per 20 µL PCR reactions. PCR reactions were performed using DyNAZYME Mastermix (Finzyme, Finland) in duplicate. PCR products were resolved on a 2% Agarose gel containing Ethidium bromide and the band intensities were determined using a Gel documentation system (UviPro platinum, Uvitec, UK). The expression of RPL-35A, a ribosomal protein, whose expression was consistent across treatments in the microarray, was used as a normalizing tool version 9.5.3.1 (Agilent Technologies) and data analysis was done using Bioconductor LIMMA package. The background-corrected raw intensity values were used for analysis. LOWESS algorithm was used to normalize the data and fold change (Fc) was calculated based on the ratio of Cy5/Cy3 (treated/untreated) intensities. For statistical analysis, Lmfit and eBayes (Empirical Bayes method) test was performed and P value correction was performed using Benjamini Hochberg method [19]. First commonly regulated genes between areca nut water extract (5H) and TGF-β was found out (using a P value<0.05 and fold change cut off of ≥1.5). To find out genes that were compromised upon areca nut treatment (5H) in presence of ALK5 inhibitor, ALK5 inhibitor array intensity was taken as control and the differentially regulated genes were identified in the 5H±ALK5 inhibitor arrays. All the microarray data have been submitted to GEO database and accession number is GSE38227.

Preparation of Areca nut extracts and fractionation

Areca nut extract preparation and fractionation were performed according to previously described methods [20,21]. Briefly thirty grams of dried and de-husked Betel nut was ground and extracted by 100 ml of de-ionized water for 4 h at 4°C with constant stirring. After filtration with a sintered glass funnel, these extracts were lyophilized and re-dissolved in de-ionized water. Insoluble components were further extracted with ethanol by the same

\[
\delta CT = CT \text{ gene} - CT \text{ RPL}
\]

\[
\delta \delta CT = \delta CT \text{ treated} - \delta CT \text{ untreated}
\]
procedure as above. All the extracts were then re-filtered through a 0.2 \( \mu \)m filter, divided into aliquots, lyophilized and stored at 4°C. For treatments, the weighed dry powder was dissolved in deionized water and stored at 270°C.

Filtered water extract samples were partitioned with dichloromethane in the ratio of 1:1 by volume. Then the water phase was collected and the impurities associated with dichloromethane were also collected (DCM phase). The partitioning with dichloromethane was repeated for 3 times and the water phase was further partitioned with ethyl acetate (1:1 by volume), which was also repeated for 3 times. The ethyl acetate extracts were collected and evaporated to dryness with vacuum rotary evaporator (Buchi Rotavapor model R-210, Switzerland) before analysis. All the three phases namely Dichloromethane (DCM phase), water (Alkaloid phase) and ethyl acetate (Polyphenol phase) were used for treating cells. To check the cross contamination between alkaloid and polyphenol extracts, LC-MS profiling of areca nut alkaloid and polyphenol fractions were performed (Figure S1). The above LC-MS profile’s retardation time and m/z values were matched with the known predominant alkaloid (Arecoline) and polyphenol (catechin) of areca nut. Arecoline peak was found to be present only in alkaloid fraction and Catechin peak was found to be present only in polyphenol fractions, highlighting the purity of fractions with respect to major alkaloids and polyphenols (Figure S2).

### Western blot analysis

Proteins were extracted from cells after DPBS wash using lysis buffer (Tris-HCl 50 mM, NaCl 150 mM, SDS 0.1%, NP-40 0.5%, and protease inhibitor cocktail, Sigma Aldrich, USA). Equal amount of protein (determined by Bradford method) extracted from cells were resolved on 12% SDS-PAGE gel, transferred to polyvinylidene difluoride membrane and subjected to immunoblot analysis. To block nonspecific binding sites, blots were incubated in 5% non-fat dry milk for 1 h followed by overnight incubation in primary antibodies at 4°C diluted according to the manufacturer's instructions [p-SMAD2, Cell Signaling Technology, Massachusetts, 3101, dilution 1:1000; Total SMAD2, Cell Signaling Technology, Massachusetts, 3162, dilution 1:1000; TGM2, Table 3. List of top 30 genes showing Up or Down regulation upon areca nut treatment on HaCaT cells.

| Up Regulated Genes | Gene Name | Genbank accession | Fold Change |
|--------------------|-----------|--------------------|-------------|
| VIM NM_003380      | 5.28      |                    |             |
| KRT16 NM_005557    | 4.12      |                    |             |
| AK021467 A_32_P49116 | 4.1       |                    |             |
| SERPINB3 NM_006919 | 4.01      |                    |             |
| SERPINB4 NM_002974 | 3.99      |                    |             |
| C8orf68 BC022082   | 3.88      |                    |             |
| AF334588 AF334588  | 3.82      |                    |             |
| AKR1C1 NM_001353   | 3.8       |                    |             |
| A_24_P610387 A_24_P610387 | 3.68 |                    |             |
| MFAP5 NM_003480    | 3.62      |                    |             |
| AKR1C3 NM_003739   | 3.61      |                    |             |
| SRPX NM_006307     | 3.54      |                    |             |
| A_24_P934592 A_24_P934592 | 3.54 |                    |             |
| FCR1A NM_002738    | 3.45      |                    |             |
| TRIM49 NM_002358   | 3.42      |                    |             |
| SCN2B NM_004588    | 3.22      |                    |             |
| GK NM_203391       | 3.14      |                    |             |
| HMox1 NM_002133    | 3.07      |                    |             |
| SERPINE2 NM_006216 | 3.03      |                    |             |
| A_32_P66987 A_32_P66987 | 3.03 |                    |             |
| A_23_P135634 A_23_P135634 | 2.98 |                    |             |
| DOCK4 NM_014705    | 2.96      |                    |             |
| A_24_P934989 A_24_P934989 | 2.89 |                    |             |
| LOC151438 AK055877 | 2.88      |                    |             |
| ACA2A NM_006111    | 2.85      |                    |             |
| MAF NM_001031804   | 2.83      |                    |             |
| ANO7 NM_001001891  | 2.83      |                    |             |
| HSPB3 NM_006308    | 2.79      |                    |             |
| BAAT NM_001701     | 2.78      |                    |             |
| VNN3 NR_028290     | 2.77      |                    |             |

| Down Regulated Genes | Gene Name | Genbank accession | Fold Change |
|----------------------|-----------|--------------------|-------------|
| CCL2 NM_002982       | 3.99      |                    |             |
| PII1 NM_025049       | 3.37      |                    |             |
| A_32_P49116 A_32_P49116 | 3.34 |                    |             |
| BIRC3 NM_001165      | 3.19      |                    |             |
| LCN2 NM_005564       | 3.16      |                    |             |
| IFI44L NM_006820     | 2.99      |                    |             |
| CLEC2B NM_005127     | 2.99      |                    |             |
| A_23_P369966 A_23_P369966 | 2.79 |                    |             |
| CA9 NM_001216        | 2.76      |                    |             |
| IFIT3 NM_001549      | 2.75      |                    |             |
| FAM72D NM_207418     | 2.72      |                    |             |
| ELF3 NM_004433       | 2.65      |                    |             |
| BE138567 BE138567   | 2.65      |                    |             |
| ASPM NM_018136       | 2.61      |                    |             |
| KIF23 NM_138555      | 2.59      |                    |             |
| APOBEC3B NM_004900   | 2.59      |                    |             |
| UBE2C NM_181803      | 2.58      |                    |             |
| LOC283711 XR_040656  | 2.58      |                    |             |
| A_23_P76480 A_23_P76480 | 2.54 |                    |             |
| BUB1B NM_001211      | 2.52      |                    |             |
| CDKN3 NM_005192      | 2.51      |                    |             |
| TPX2 NM_012112       | 2.5       |                    |             |
| CENPA NM_001809      | 2.47      |                    |             |
| NMU NM_006681        | 2.47      |                    |             |
| TROAP NM_005480      | 2.47      |                    |             |
| BIRC5 NM_001012271  | 2.44      |                    |             |
| CENPE NM_001813      | 2.41      |                    |             |
| A_32_P188921 A_32_P188921 | 2.41 |                    |             |
| NUF2 NM_145697       | 2.41      |                    |             |
| SPAG5 NM_006461      | 2.4       |                    |             |

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R&D SYSTEMS, MAB3542, dilution 1:500; β-Actin, Sigma-Aldrich, C2206, dilution 1:2000). This was followed by incubation with horseradish peroxidase-conjugated secondary antibody (Anti-rabbit/mouse HRP conjugated secondary antibody Sigma-Aldrich, St. Louis, MO, USA, dilution 1:2000). Proteins were visualized with a chemiluminescence detection system (Super Signal West Femto Chemiluminescent Substrate, Thermo Fisher Scientific Inc. USA, 34095) and subsequent exposure to X-ray film.

**TGF-β2 Immunoassay**

TGF-β2 in the spent medium of areca nut treated cells were measured using commercially available Quantikine human TGF-β2 immunoassay kit (R&D Systems, Minneapolis, MN, USA). Briefly, HaCaT cells spent medium untreated/treated with different concentrations of areca nut were activated by using Hydrochloric acid (1 N) for 10 min and later mixed with HEPES (0.5 M)/NaOH (1.2 N) buffer followed by dilution in calibrator diluent. Standards and samples are pipetted into the wells pre-coated with TGF-β2 monoclonal antibody and allowed to bind for 2 hrs at ambient temperature. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β2 was added to the wells and washed with buffer to remove any unbound antibody-enzyme reagent. Following this, substrate solution was added to the wells and colour development was allowed for 20 min, stopped by adding stop buffer and the intensity of the colour was measured at 450 nm after wavelength correction at 570 nm. Concentration of TGF-β2 was calculated from the standard curve.

**Total collagen staining using Direct Red 80**

Direct Red 80 was purchased from Sigma-Aldrich, USA and the staining protocol followed has been described earlier [22]. Direct Red 80 dye was dissolved in saturated aqueous picric acid at a concentration of 100 mg/100 ml. Bouin’s fluid (for cell fixation) was prepared by mixing 15 ml saturated aqueous picric acid with 5 ml 35% formaldehyde and 1 ml glacial acetic acid. After treatment of

### Table 4. List of top 30 commonly regulated genes showing Up or Down regulation by TGF-β and areca nut treatment on HaCaT cells.

| Gene Name | Genbank accession | TGF-β | SH | Gene Name | Systematic Name | TGF-β | SH |
|-----------|-------------------|-------|----|-----------|----------------|-------|----|
| PDPN      | NM_006474         | 15.91 | 2.41| UBE2C     | NM_181803      | 17.87 | 2.58|
| MAF       | NM_001031804      | 13.44 | 2.83| BIRC5     | NM_001012271   | 16.70 | 2.30|
| ACA2A     | NM_006111         | 13.40 | 2.85| CEP55     | NM_018131      | 15.50 | 2.00|
| MMP13     | NM_002427         | 13.27 | 1.52| ASPM      | NM_018136      | 15.28 | 2.61|
| A_23_P123234 | A_23_P123234   | 10.49 | 2.21| DEPDC1    | NM_017779      | 14.72 | 2.64|
| SERPINE1  | NM_000602         | 9.68  | 1.45| NDC80     | NM_006101      | 13.71 | 1.91|
| CST6      | NM_001323         | 9.54  | 2.37| PBK       | NM_018492      | 13.64 | 1.91|
| FCRLA     | NM_032738         | 8.67  | 3.45| BUB1      | NM_004336      | 13.35 | 1.83|
| PDZK1     | NM_002614         | 8.25  | 1.81| NCAF      | NM_022346      | 13.29 | 1.74|
| VIM       | NM_003380         | 8.16  | 5.28| SPAG5     | NM_006461      | 12.71 | 2.40|
| A_32_P115518 | A_32_P115518  | 8.04  | 1.50| CDC5      | NM_080668      | 12.70 | 1.84|
| PRSS23    | NM_007173         | 7.98  | 1.74| CDT1      | NM_030928      | 12.64 | 1.65|
| VASN      | NM_138440         | 7.78  | 1.78| TTK       | NM_003318      | 12.57 | 2.09|
| BAMBI     | NM_012342         | 7.67  | 1.78| MND1      | NM_032117      | 12.49 | 1.73|
| A_23_P87421 | A_23_P87421    | 7.50  | 1.51| NUF2      | NM_145697      | 11.98 | 2.41|
| KRT16     | NM_005557         | 7.39  | 4.12| BUB1B     | NM_001211      | 11.74 | 2.52|
| TGM2      | NM_004613         | 7.31  | 1.81| KIF23     | NM_138555      | 11.44 | 2.59|
| HILS1     | NR_024193         | 7.26  | 1.50| FAM72D    | NM_207418      | 11.41 | 2.72|
| TPM1      | NM_000366         | 7.25  | 1.62| SKA3      | BC013418       | 11.35 | 1.74|
| WNT5B     | NM_030775         | 7.08  | 1.53| HMG2B     | NM_002129      | 11.27 | 2.37|
| MFAP5     | NM_003480         | 6.68  | 3.62| KIF18B    | BC044933       | 10.67 | 2.35|
| SERPINE2  | NM_006216         | 6.42  | 3.03| FAM83D    | NM_030919      | 10.29 | 2.00|
| ALOX5     | NM_00698         | 6.37  | 2.13| TOP2A     | NM_001067      | 10.06 | 2.04|
| A_32_P52414 | A_32_P52414   | 6.15  | 2.19| CCNA2     | NM_001237      | 9.90  | 2.14|
| LOC151438 | AK055877         | 6.12  | 2.88| KIF2C     | NM_006845      | 9.88  | 2.02|
| A_24_P633825 | A_24_P633825  | 6.11  | 2.18| CENPA     | NM_001809      | 9.83  | 2.47|
| HSPB3     | NM_006308         | 5.83  | 2.79| CIT       | NM_007174      | 9.73  | 1.89|
| DACT1     | NM_016651         | 5.56  | 1.51| CDC2      | NM_001786      | 9.69  | 1.89|
| SLITRK6   | NM_032229         | 5.53  | 1.67| FAM64A    | NM_190193      | 9.64  | 1.86|
| SPOCK1    | NM_004598         | 5.47  | 2.42| LMNB1     | NM_005573      | 9.42  | 1.95|

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hGF cells, cells were washed with PBS, fixed with 1 ml Bouin’s fluid for 1 hr followed by washing with PBS for 15 min. Cells were air dried before adding 1 ml Direct Red dye reagent for 1 h under mild shaking on a microplate shaker. Later on dye solution was removed followed by washing in 0.01 N hydrochloric acid to remove excess dye. The stained cells were photo documented before dissolving the stain. For quantitation, 1 ml of 0.1 N NaOH was added to 35 mm culture dishes followed by incubation for 30 min at room temperature on microplate shaker. The dye solution’s optical density (OD) was measured by spectrophotometer (Bio-Rad, SmartSpec-3000 Spectrophotometer) at 550 nm against 0.1 N NaOH as a blank and OD/10^5 cells were plotted.

Statistical analysis

Statistical significance was determined using one-way analysis of variance (ANOVA) among different treatments. All treatments were compared to untreated and the observed significance levels from multiple comparisons were made using the Bonferroni’s Multiple Comparison Test with P≤0.05 indicating significance (P value≤0.01, ≤0.001 and ≤0.0001 are indicated by *, **, and *** respectively).

Results

Areca nut induces TGF-β signaling in epithelial cells

In order to study the areca nut effects on epithelial cells, gene expression profiling was performed using Agilent whole human genome 4×44 K arrays with the RNAs extracted from HaCaT cells treated with areca nut water extract (5 μg/ml; 5H). 1331 genes were found to be differentially expressed following treatment with areca nut water extract (P≤0.05, fold change ≥1.5). KEGG pathway analysis revealed regulation of genes categorized in several pathways and among them prominent were pathways in cancer, cell cycle, cytokine-cytokine receptor interaction, Wnt signaling pathway etc (Table 2). Interestingly many of these genes are targets of TGF-β signaling [23], which include TGF-β2, SMAD3, FN1, MMP1, MMP2, MMP9, CCNE1 etc. In a previous microarray study on differentially expressed genes in OSF, we and others proposed activation of TGF-β pathway as a major cause of OSF pathogenesis. In view of this, and regulation of TGF-β pathway genes by areca nut extract, we hypothesized that areca nut actions could be through the activation of TGF-β signaling. To test this, we compared expression profile of differentially regulated genes in HaCaT cells by areca nut water extract and TGF-β. Treatment of HaCaT cells by areca nut extract and TGF-β resulted in the regulation of 1331 and 3365 genes respectively. Of areca nut induced genes, 853 genes (64%) matched with TGF-β regulated genes (Figure 1A & Ci, Table S1). List of top 30 genes up and down regulated by areca nut extract along with commonly regulated genes by areca nut extract and TGF-β are shown in table 3 and 4, respectively. A high match in differentially regulated genes by areca nut extract and TGF-β suggests a possible activation of TGF-β signaling by areca nut extract. To test this, HaCaT cells were treated with areca nut water extract in the presence of TGFRI inhibitor (ALK5 inhibitor). UN-untreated, WS- water supernatant, POL- Polyphenol supernatant, DCM- Dichloromethane fraction.

Figure 3. Alkaloid and Polyphenol fractions of Areca nut induce TGF-β signaling in HaCaT cells. Treatment of HaCaT cells with both the Alkaloid and Polyphenol fractions of areca nut water extract induced TGF-β signaling (p-SMAD2) and its down-stream target TGM2 as shown by the western blot (Figure 3A). Expression of TGF-β down-stream targets were also studied by Real Time PCR (Figure 3B) and semi quantitative PCR (Figure 3C). Induction of genes by alkaloid and polyphenol fractions of areca nut was compromised in presence of TGFRI inhibitor (ALK5 inhibitor). (UN-untreated, WS- water supernatant, POL- Polyphenol supernatant, DCM- Dichloromethane fraction). doi:10.1371/journal.pone.0051806.g003
the role of TGF-β in areca nut actions. Hierarchical cluster analysis of the differentially regulated genes following the above treatments, confirmed common gene expression profiles by areca nut and TGF-β (Figure 1B). The above results demonstrate areca nut inducing/activating TGF-β signaling in epithelial cells. To confirm this we treated HaCaT cells with areca nut water extract in the presence or absence of ALK5 inhibitor and activation of TGF-β pathway was studied. By western blot analysis, we observed phosphorylation of SMAD2 (an effector of TGF-β signaling) after treatment with areca nut water extract (Figure 2A). Also, protein expression of TGM2 (a TGF-β induced gene) was found to be induced by areca nut extracts, suggesting activation of TGF-β pathway by areca nut extract. Further, by qPCR, expression of TGF-β target genes (TGM2, TMEPAI, THBS1 and TGFBI) was found to be up regulated following treatment with areca nut extracts (Figure 2B). All these effects of areca nut were compromised in the presence of ALK5 inhibitor (Figure 2C and D).

Figure 4. Both the pure alkaloids and polyphenols of areca nut induce TGF-β signaling. Human keratinocytes (HaCaT) cells were serum deprived for 24 hours and treated with areca nut extracts, pure Alkaloids and Polyphenols for 48 hrs. A, shows pSMAD2 by western blot and B, expression of BMP7 by real time PCR; C & D, TGF-β2 expression by real time PCR; E, TGF-β2 protein estimation by ELISA; F, THBS1 expression by real time PCR; G & H, THBS1 western blots. The treatments are depicted in the respective figures. UN- untreated, Arecoline (400 μM), Arecaidine (1000 μM), Gувачин (1000 μM), Catechin (170 μM), Tannin (6 μM), TGF-β, WS- water supernatant, POL- Polyphenol supernatant.
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Areca nut alkaloids and polyphenols induce TGF-β signaling
In order to find out areca nut components responsible for inducing TGF-β signaling, fractionation of areca nut water extract was performed as described in materials and methods. Upon treatment of areca nut fractions (alkaloids and polyphenols) on HaCaT cells, both the alkaloid and polyphenol fractions induced phosphorylation of SMAD2, expression of TGF-β down-stream targets – TGM2, THBS1, TGFB1 and TIMP1 which were compromised in the presence of ALK5 inhibitor (Figure S3A–C). Similar results were observed in foreskin keratinocytes where treatment of both the areca nut fractions (alkaloids and polyphenols) were able to induce TGF-β down-stream targets which gets compromised in presence of TβRI inhibitor (Figure S3E–H). Constituents of areca nut alkaloid and polyphenol fractions are well defined [21]. Arecoline is the most predominant alkaloid (~0.2%) followed by Arecaidine, Gувачин etc and
Catechin and Tannin are the most predominant polyphenols present along with minor polyphenols like Epicatechin, Gallic acid etc [21]. In order to determine which alkaloid or polyphenol of areca nut induce TGF-β signaling, we treated HaCaT cells with pure alkaloids (Arecoline, Arecaidine and Guvacine) and pure polyphenols (Catechin and Tannin). Similar to water and polyphenol fractions of areca nut extract; arecoline, arecaidine, guvacine, catechin and tannin also induced phosphorylation of SMAD2, expression of down-stream target TGM2 (Figure 4A). In contrast, the anti-fibrogenic cytokine BMP7 [10] was found to be down-regulated following the above treatments (Figure 4B). In addition, treatment of HaCaT cells with water and ethanol extracts, and pure compounds resulted in the induction of TGF-β2 RNA and protein (Fig. 4C–E). Also, THBS1 RNA and protein were induced by above treatments (Figure 4F–H). Therefore induction of TGF-β signaling by areca nut and its constituent in epithelial cells is by inducing both ligand (TGF-β2) and its activator, THBS1.

Areca nut does not induce TGF-β signaling in fibroblast cells

During chewing of betel quid, epithelium is first exposed to areca nut components and later it gets diffused into sub-mucosal region. Therefore, areca nut components may affect both epithelial cells as well as connective tissue consisting of fibroblasts that may be important for the pathogenesis of OSF. Hence we also studied the actions of areca nut extracts on human gingival fibroblasts. In contrast to its action on epithelial cells, areca nut water extract did not induce p-SMAD2 and its down-stream targets in human gingival fibroblast (hGF) cells (Figure S4A & B). Similarly, treatment of hGF cells with alkaloid, DCM and polyphenol fractions of areca nut water extract did not show any induction of TGF-β target genes (Figure S4C). These observations suggest that areca nut influence on fibroblasts may not be as profound as compared to epithelial cells in the induction of pro fibrogenic cytokines.

Areca nut potentiates TGF-β action on hGF cells

In response to areca nut components, TGF-β induced by the epithelium can diffuse into connective tissues and effect fibrotic changes in the sub-mucosa. Since areca nut did not activate TGF-β pathway in fibroblasts, we studied the combined actions of areca nut and TGF-β on fibroblasts. Interestingly, upon exposure of fibroblast cells (both hGF and FF) to areca nut extract in the presence of TGF-β, there was enhanced expression of TGF-β target genes, THBS-1, TMEPAI, TGFBI and TGM-2 (Figure 5A–D). Therefore areca nut components may potentiate TGF-β action on fibroblast cells.
A–D & Figure S5 A–D). This suggests potentiation of TGF-$\beta$ action by areca nut water extract on fibroblasts. Furthermore, in response to areca nut and TGF-$\beta$, the expression of $\alpha$-SMA and collagen isoforms (Col1A1, 1A2 and Col3A1) was found to be synergistic and additive, respectively (Figure 5 E–H). Similar results were also observed in FF cells upon treatments of areca nut and TGF-$\beta$ (Figure S5 E–H). This additive effect of TGF-$\beta$ and areca nut on collagen expression was also observed for total collagen protein as revealed by Direct Red dye staining in both hGF and FF cells (Figure 5 I, J & Figure S5 I).

**Discussion**

Etiology of OSF is highly complicated and involves intrinsic and extrinsic factors including habit of betel quid chewing coupled with predisposition of the subjects for developing OSF [24]. Several etiological factors have been proposed but arecoline present in the betel nut has been shown to be the most important alkaloid implicated in OSF development [25]. In previous reports including ours, up-regulation and activation of TGF-$\beta$ signaling in OSF has been demonstrated [9,10]. This activation of TGF-$\beta$ involves up-regulation of $\alpha V\beta 6$ integrins by arecoline. The above findings highlight the role of arecoline in TGF-$\beta$ activation and a central role for TGF-$\beta$ pathway in the development of OSF. But the amount of arecoline needed to induce TGF-$\beta$ signaling and its down-stream targets was much higher (50 $\mu$g/ml or 400 $\mu$M) than the amount of areca nut water extract (2.5 $\mu$g/ml). This led us to speculate that areca nut constituents other than arecoline could also be involved in inducing TGF-$\beta$ signaling in epithelial cells.

We demonstrate for the first time that both water and alcohol extracts of areca nut induce TGF-$\beta$ signaling in epithelial cells as shown by increased levels of p-SMAD2. This could be due to induction of TGF-$\beta$ ligand (TGF-$\beta$2) and its activator THBS-1 leading to activation of TGF-$\beta$ pathway. Accordingly, areca nut induced expression shares a large number of genes (64%) regulated by TGF-$\beta$. In the TGF-$\beta$ signaling cascade, TGF-$\beta$ ligands (1–3) bind to $T\beta R I$ receptor that recruits $T\beta R I$ (ALK5) leading to phosphorylation of ALK5 that facilitates SMAD2/3 phosphorylation. Activated SMAD2 or SMAD3 demonstrates activation of TGF-$\beta$ signaling. Inhibition of ALK5 leads to abrogation of SMAD 2/3 phosphorylation [18]. Therefore we used an ALK5 inhibitor to demonstrate TGF-$\beta$’s role in areca nut actions. As expected, SMAD2 phosphorylation following areca nut treatment was completely abolished in the presence of ALK5 inhibitor and consequently majority of areca nut regulated genes (57%) were also compromised. This suggests that areca nut regulated gene expression in HaCaT cells involves TGF-$\beta$ signaling. It is also evident that there are genes that are regulated by areca nut independent of TGF-$\beta$ signaling. As of now, the role of these genes in the OSF pathogenesis is not known.

We also establish that in addition to arecoline, other alkaloids such as arecardine and guvacine are able to induce TGF-$\beta$ signaling. Most importantly, both the predominant polyphenols of areca nut (Catechin and Tannin) were found to be potent inducers of TGF-$\beta$ signaling. This is a very profound finding that the alkaloids, other than arecoline and predominant polyphenols of areca nut are potent inducers of pro fibrogenic cytokine, TGF-$\beta$. Polyphenols constitute 11–17.8% in mature areca nut compared to its predominant alkaloid arecoline (0.2%) [21]. This higher
percentage of polyphenols in areca nut could play a dominant role in inducing pro-fibrogenic (TGF-β) pathway and consequently, OSF.

Our studies revealed that areca nut has very little direct influence on the expression of pro fibrogenic genes in the fibroblasts, where the actual disease process occurs. However, our data advocates that TGF-β induced in the epithelium by areca nut acts on the fibroblasts in a pro fibrogenic manner by the induction of matrix components such as collagens. This is also supported by our previous results showing hGF cells if cultured in HaCaT spent medium treated with arecoline, induced collagen isoforms which gets neutralized by LAP (Latency Associated peptide, a TGF-β antagonist) [26]. Most interestingly, although areca nut has minimal influence on fibroblasts, it synergizes with TGF-β in activating fibroblast cells. This is very important in the context of OSF manifestation in susceptible individuals. Chronic areca nut chewing may trigger pro-inflammatory/fibrotic factors from epithelium and these factors may synergize with areca nut leading to fibroblast activation, where the disease process occurs (Figure 6).

In conclusion, in this manuscript we propose a causative role for areca nut components in triggering a pro fibrogenic cascade involving TGF-β pathway from the epithelial cells influencing the underlying sub mucosa for a fibrotic response. For the first time we show the potent induction of TGF-β pathway by polyphenols and alkaloids in addition to arecoline present in areca nut extract.

Supporting Information

Figure S1 Liquid chromatography of alkaloid and polyphenol fractions. Areca nut water extract was partitioned into two phases namely, Ethyl acetate (Polyphenol) fraction and Water supernatant (Alkaloid) fraction. To asses the purity of fractions both the above fractions were separated in HPLC. Figure S1 A&B shows the retardation profile of the two fractions which does not match with each other highlighting both have different components.

(TIF)

Figure S2 Identification of major components of alkaloid and polyphenol fractions by LC–MS. In order to see the distribution of areca nut major alkaloids and polyphenols in the Ethyl acetate and water supernatant fractions, MS Spectrum of pure Arecoline and pure catechin were matched with the above two fractions. Arecoline MS profile (m/z- 79, 156.76) matched with water supernatant (Figure S2 A) but not with ethyl acetate fraction. Similarly Catechin MS profile (m/z 279, 579 and 867) matched with ethyl acetate fraction (Figure S2 B) but not with water supernatant, highlighting no cross contamination of major alkaloid and polyphenol in the fractionation.

(TIF)

Figure S3 Areca nut does not induce TGF-β signaling in fibroblast cells. Human gingival fibroblast (hGF) cells were treated for 72 hours with areca nut water (H) or ethanol (Et) extract 2.5, 5 μg/ml (2.5H/5H & 2.5Et/5Et) in serum free medium for 48 hours. A-D) qRT-PCR analysis showing expression of TGF-β down-stream target genes TGM2, TMEPAI, THBS1, TGFBI and ALK5 (TβRI) inhibitor (SB 431542) reverses the Areca nut induced expression of the above genes. Treatment of human Foreskin keratinocytes cells with both the Alkaloid and Polyphenol fractions of areca nut water extract induced TGF-β down-stream target as shown by Real Time PCR (Figure S4E-H) and induction of genes by alkaloid and polyphenol fractions of areca nut was compromised in presence of TβRI inhibitor. UN-Un-treated, 2.5H & 5H- 2.5 and 5 μg/ml Areca nut water extract, 2.5 Et, 5Et- 2.5 and 5 μg/ml, ALK5-ALK5 inhibitor, T-TGF-β, WS- water supernatant, POL- Polyphenol supernatant, DCM- Dichloromethane fraction.

(* = P<0.0001, compared to untreated).

(TIF)

Figure S4 Areca nut induces TGF-β signaling in foreskin keratinocytes through TGF-β. Human Foreskin keratinocytes cells were serum starved for 24 hours and treated with either areca nut water (H) or ethanol (Et) extracts 2.5, 5 μg/ml (2.5H/5H & 2.5Et/5Et) in serum free medium for 48 hours. A-D) qRT-PCR analysis showing expression of TGF-β down-stream target genes TGM2, TMEPAI, THBS1, TGFBI and ALK5 (TβRI) inhibitor (SB 431542) reverses the Areca nut induced expression of the above genes. Treatment of human Foreskin keratinocytes cells with both the Alkaloid and Polyphenol fractions of areca nut water extract induced TGF-β down-stream target as shown by Real Time PCR (Figure S4E-H) and induction of genes by alkaloid and polyphenol fractions of areca nut was compromised in presence of TβRI inhibitor. UN-Un-treated, 2.5H & 5H- 2.5 and 5 μg/ml Areca nut water extract, 2.5 Et, 5Et- 2.5 and 5 μg/ml, ALK5-ALK5 inhibitor, T-TGF-β, WS- water supernatant, POL- Polyphenol supernatant, DCM- Dichloromethane fraction.

(* = P<0.0001, compared to untreated).

(TIF)

Figure S5 Areca nut potentiates TGF-β action on FF cells. Human foreskin fibroblast (FF) cells were treated for 72 hours with areca nut water extract (5H- 5 μg/ml) and or TGF-β (5 ng/ml) after 24 hrs of serum deprivation in 0.2% serum and the expression of genes were looked at by Real-Time PCR (Figure S5 A-H). FF cells were treated with areca nut water extract and or TGF-β for 3 days and stained with “Direct Red 80” for total collagen. Figure S5 I shows the quantitation of the Direct Red staining as measured at 550 nm.

(TIF)

Table S1 List of commonly regulated genes by Areca nut water extract (5H) and TGF-β (p≤0.05; Fold Change 1.5).

(XLS)

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Author Contributions

Conceived and designed the experiments: IK PK. Performed the experiments: IK IP SN. Analyzed the data: IK NK. Contributed reagents/materials/analysis tools: PK. Wrote the paper: IK PK.

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