Chronic exposure to chewing tobacco selects for overexpression of stearoyl-CoA desaturase in normal oral keratinocytes

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Chronic exposure to chewing tobacco selects for overexpression of stearoyl-CoA desaturase in normal oral keratinocytes

Vishalakshi Nanjappa1,2, Santosh Renuse1,2, Gajanan J Sathe1,3, Remya Raja1, Nazia Syed1,4, Aneesha Radhakrishnan1,4, Tejaswini Subbannayya1,2, Arun Patil1,5, Ariusudar Marimuthu1, Nandini A Sahasrabuddhe1, Rafael Guerrero-Preston6, Babu L Somani1, Bipin Nair2, Gopal C Kundu7, T Keshava Prasad1,2,8, Joseph A Califano6,9, Harsha Gowda1,8, David Sidransky6, Akhilish Pandey10,11,12, and Aditi Chatterjee1,8,*

1Institute of Bioinformatics; International Technology Park; Bangalore, India; 2Amrita School of Biotechnology; Amrita University; Kollam, India; 3Manipal University; Madhav Nagar; Manipal, India; 4Department of Biochemistry and Molecular Biology; Pondicherry University; Puducherry, India; 5School of Biotechnology; KIIT University; Bhubaneswar, India; 6Department of Otolaryngology-Head and Neck Surgery; Johns Hopkins University School of Medicine; Baltimore, MD USA; 7National Center for Cell Science (NCCS); NCCS Complex; Pune, India; 8YU-IOB Center for Systems Biology and Molecular Medicine; Yenepoya University; Mangalore, India; 9Milton J. Dance Head and Neck Center; Greater Baltimore Medical Center; Baltimore, MD USA; 10McKusick-Nathans Institute of Genetic Medicine; Johns Hopkins University School of Medicine; Baltimore, MD USA; 11Department of Biological Chemistry; Johns Hopkins University School of Medicine; Baltimore, MD USA; 12Department of Pathology; Johns Hopkins University School of Medicine; Baltimore, MD USA

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Abbreviations: iTRAQ, Isobaric tags for relative and absolute quantitation; SCX, Strong cation exchange chromatography

Chewing tobacco is a common practice in certain socio-economic sections of southern Asia, particularly in the Indian subcontinent and has been well associated with head and neck squamous cell carcinoma. The molecular mechanisms of chewing tobacco which leads to malignancy remains unclear. In large majority of studies, short-term exposure to tobacco has been evaluated. From a biological perspective, however, long-term (chronic) exposure to tobacco mimics the pathogenesis of oral cancer more closely. We developed a cell line model to investigate the chronic effects of chewing tobacco. Chronic exposure to tobacco resulted in higher cellular proliferation and invasive ability of the normal oral keratinocytes (OKF6/TERT1). We carried out quantitative proteomic analysis of OKF6/TERT1 cells chronically treated with chewing tobacco compared to the untreated cells. We identified a total of 3,636 proteins among which expression of 408 proteins were found to be significantly altered. Among the overexpressed proteins, stearoyl-CoA desaturase (SCD) was found to be 2.6-fold overexpressed in the tobacco treated cells. Silencing/inhibition of SCD using its specific siRNA or inhibitor led to a decrease in cellular proliferation, invasion and colony forming ability of not only the tobacco treated cells but also in a panel of head and neck cancer cell lines. These findings suggest that chronic exposure to chewing tobacco induced carcinogenesis in non-malignant oral epithelial cells and SCD plays an essential role in this process. The current study provides evidence that SCD can act as a potential therapeutic target in head and neck squamous cell carcinoma, especially in patients who are users of tobacco.

Introduction

Consumption of tobacco continues to be one of the major established etiological factors in the pathogenesis of head and neck squamous cell carcinoma (HNSCC).1,2 According to the report published by the WHO, every year approximately 6 million people worldwide are killed due to tobacco consumption.3 Tobacco is mainly consumed worldwide in the form of manufactured cigarettes and even smoked as ‘bidis’, cigars and water-pipes.4 Another form in which tobacco is consumed includes smokeless or chewing tobacco. India houses the highest number of chewing tobacco users, which accounts for 80% of global tobacco chewers. In India, more than 50% of oral cancer cases are estimated due to use of chewing tobacco.4

Chewing tobacco is associated with the development of cancers of the oral cavity, esophagus, stomach and pancreas.5,6 In the year 2007, the International Agency for Research on Cancer (IARC) classified chewing tobacco as a human carcinogen.7 Chewing tobacco contains several compounds including tobacco-specific N-nitrosamines (TSNA), N’-nitrosornornicotine (NNN), and 4(methylisoxazolino)-1-((3-pyridyl)-1-butanone (NNK) which are shown to be carcinogenic.8,9 Though several studies have shown the association between chewing tobacco and cancer, the underlying cellular and molecular dynamics that lead to cancer development and metastasis is not well characterized. It is intuitive to consider that the mechanisms of action of chewing tobacco to be similar to that of cigarette smoke. However, it has been reported that there are significant differences in
pro-carcinogenic and carcinogenic agents present in chewing tobacco because of which the mode of action might vary significantly. Current studies have demonstrated the acute effects of chewing tobacco in cells. A study by Wang Y et al., demonstrated the proliferation of normal human epidermal keratinocytes induced by chewing tobacco extract when treated for 3 days. Studies by other groups have shown that treatment of AMOL-III cells (established from a leukoplakia patient), with chewing tobacco for 48h induced alterations in genes involved in key biological processes such as cell cycle regulation, cell-cell adhesion and DNA methylation. Validation of these genes on oral premalignant lesions (OPLs) and oral squamous cell carcinoma (OSCC) tissues showed similar pattern of expression. The same group also reported activation of nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) pathways in chewing tobacco induced oral carcinogenesis. It is known that carcinogenic effect of chewing tobacco in oral cancer is through chronic exposure and not by acute exposure. Though these studies have identified few of the molecules by which chewing tobacco may exert its effects, but till date, there has been no study on the chronic effects of chewing tobacco in oral keratinocytes even though chewing tobacco is one of the primary risk factors for head and neck cancers.

**Figure 1.** Chronic exposure to chewing tobacco increases proliferation and invasive property of oral keratinocytes. (A) Growth curve for OKF6/TERT1 and OKF6/TERT1-Tobacco cells. OKF6/TERT1-Tobacco cells showed higher proliferation rate than the parental cells. (B) Invasion assay: OKF6/TERT1 chronically treated with chewing tobacco acquired invasive ability. (C) OKF6/TERT1 chronically treated with chewing tobacco showed an increase in Bcl-xL/Bax ratio.

**Figure 2.** Workflow employed to identify the proteins differentially expressed in response to chewing tobacco. Proteins from OKF6/TERT1 and OKF6/TERT1-Tobacco cells were isolated and quantified. Equal amount of proteins from each condition was subjected to in-solution trypsin digestion. Peptides from OKF6/TERT1 cells were labeled with iTRAQ reagents 114 and 115 and those from OKF6/TERT1-Tobacco cells were labeled with 116 and 117 iTRAQ labels. The samples were pooled and subjected to SCX fractionation, followed by mass spectrometry-based proteomic analysis.
In this study, we chronically treated normal oral keratinocytes (OKF6/TERT1) with chewing tobacco to model tobacco chewing effects. We found that chronic exposure to chewing tobacco led to increased cellular proliferation and induced invasive ability in the non-invasive oral keratinocytes. To understand the molecular mechanisms of the insult imparted by chewing tobacco, we studied the proteomic profile of the OKF6/TERT1 cells chronically exposed to chewing tobacco compared to untreated cells. Quantitative mass spectrometry-based proteomic analysis resulted in the identification of 408 proteins, which were differentially expressed in the cells chronically exposed to chewing tobacco. The dysregulated proteins included overexpression of proteins involved in the stimulation of cell growth and cell cycle regulation.

Tumor cells exhibit high cellular proliferation rate than the normal cells. This process requires the formation of new membranes which in turn requires the synthesis of lipids/fatty acids to maintain membrane fluidity.\textsuperscript{16} Saturated fatty acids produced as a result of glycolysis are converted to monounsaturated fatty acids, which serve as the components of cell membrane. The ratio of saturated to monounsaturated fatty acids is tightly regulated in cells. Any imbalance in this ratio affects a wide range of cellular functions leading to either uncontrolled cellular proliferation or cellular senescence and death.\textsuperscript{17} Stearoy-CoA desaturase (SCD), a delta-9-desaturase, plays a central role in the \textit{de novo} synthesis of monounsaturated fatty acids from saturated fatty acids. The preferred substrates of SCD include palmitic and stearic acids, which are converted to palmitoleate and oleate, respectively.\textsuperscript{18} In the absence of SCD, palmitic acid accumulates in the cells, which is toxic to mitochondria and endoplasmic reticulum and induces apoptosis.\textsuperscript{19,20} Literature evidence suggests SCD as a potential target to block cellular proliferation and invasion in cancer.\textsuperscript{21-23} However, the role of SCD in HNSCC remains unexplored. In this study, SCD was 2.6-fold overexpressed in cells chronically treated with tobacco and we have assessed the potential of SCD as a novel therapeutic target in head and neck cancer.

**Results**

**Chronic exposure to chewing tobacco increases proliferation and invasive property of oral keratinocytes**

Non-neoplastic oral keratinocytes, OKF6/TERT1, were treated at varying concentrations of chewing tobacco extract ranging from 0–10% to determine the optimum concentration for chronic treatment (data not shown). The highest concentration with which the cells could be treated chronically was 1%. Cells treated at higher concentrations of chewing tobacco (>1%) underwent apoptosis/necrosis within days of treatment (data not shown). After 3 months of chronic treatment, we observed a change in the invasive property of the oral keratinocytes. The non-invasive cells exhibited signs of invasion (data not shown). The chronic treatment was continued for a total of 6 months before the daughter cells (OKF6/TERT1 cells chronically treated with chewing tobacco, henceforth referred to as OKF6/TERT1-Tobacco) were assessed for proliferation and invasion ability. We observed a significant increase in cellular proliferation of the tobacco treated cells compared to the untreated cells (Fig. 1A). \textit{In vitro} invasion assay using Matrigel showed that the non-invasive OKF6/TERT1 cells had acquired invasive property upon chronic tobacco treatment and more that 80% of the cells had invaded the Matrigel coated PET membrane (Fig. 1B).

**Chewing tobacco induces the expression of survival proteins**

It is established that in the presence of genotypic insult, cancer cells escape cell death by regulating the expression of anti-apoptotic and pro-apoptotic genes.\textsuperscript{24} As the chewing tobacco treated cells showed enhanced cellular proliferation and invasion compared to the normal oral keratinocytes, we next examined the expression of BCL-2 family proteins in response to chewing tobacco. Western blot analysis showed an increase in expression of both BCL-xL and BCL-2 along with decreased expression of Bax in the OKF6/TERT1-Tobacco cells compared to the parental cells (Fig. 1C).

**Table 1.** Partial list of molecules differentially expressed in OKF6/TERT1 cells chronically treated with chewing tobacco compared to untreated cells.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Tobacco treated/untreated (fold change)</th>
<th>Associated biological processes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERH</td>
<td>Enhancer of rudimentary homolog</td>
<td>7.1</td>
<td>Cell cycle progression\textsuperscript{53}</td>
</tr>
<tr>
<td>ERGIC3</td>
<td>ERGIC and golgi 3</td>
<td>3.8</td>
<td>Regulation of cell growth\textsuperscript{54}</td>
</tr>
<tr>
<td>M1567</td>
<td>Marker of proliferation</td>
<td>2.8</td>
<td>Cell cycle control\textsuperscript{55}</td>
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<tr>
<td>TIMP1</td>
<td>TIMP metallopeptidase inhibitor 1</td>
<td>2.7</td>
<td>Cell growth and/or maintenance\textsuperscript{56}</td>
</tr>
<tr>
<td>SCD</td>
<td>Stearoyl-CoA desaturase</td>
<td>2.6</td>
<td>Fatty acid metabolism\textsuperscript{14}</td>
</tr>
<tr>
<td>Cagar1</td>
<td>cell division cycle and apoptosis regulator 1</td>
<td>2.6</td>
<td>Regulation of cell proliferation\textsuperscript{57}</td>
</tr>
<tr>
<td>FLNB</td>
<td>Filamin B, β</td>
<td>2.0</td>
<td>Cell proliferation\textsuperscript{38}</td>
</tr>
<tr>
<td>EPS8</td>
<td>Epidermal growth factor receptor pathway substrate 8</td>
<td>2.0</td>
<td>Cell communication; Signal transduction\textsuperscript{58}</td>
</tr>
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<td>S100A14</td>
<td>S100 calcium binding protein A14</td>
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<td>Chemotaxis\textsuperscript{59}</td>
</tr>
<tr>
<td>HSPA5</td>
<td>Heat shock 70kDa protein 5</td>
<td>1.6</td>
<td>Regulation of apoptotic process\textsuperscript{60}</td>
</tr>
<tr>
<td>ENO1</td>
<td>Enolase 1</td>
<td>0.5</td>
<td>Regulation of cell growth\textsuperscript{61}</td>
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<tr>
<td>TAGLN2</td>
<td>Transgelin 2</td>
<td>0.5</td>
<td>Tumor suppressor\textsuperscript{52}</td>
</tr>
<tr>
<td>PLIN3</td>
<td>Perilipin 3</td>
<td>0.5</td>
<td>Transport\textsuperscript{53}</td>
</tr>
<tr>
<td>KIF13A</td>
<td>Kinesin family member 13A</td>
<td>0.5</td>
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</tr>
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<td>Cadherin 13</td>
<td>0.4</td>
<td>Regulation of cell proliferation\textsuperscript{65}</td>
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<tr>
<td>PKM</td>
<td>pyruvate kinase, muscle</td>
<td>0.4</td>
<td>programmed cell death\textsuperscript{16}</td>
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<tr>
<td>S100A11</td>
<td>S100 calcium binding protein A11</td>
<td>0.3</td>
<td>Tumor suppressor\textsuperscript{67}</td>
</tr>
<tr>
<td>TENM2</td>
<td>Teneurin transmembrane protein 2</td>
<td>0.3</td>
<td>Cell adhesion\textsuperscript{68}</td>
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<tr>
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<td>Programmed cell death 4</td>
<td>0.3</td>
<td>Tumor suppressor\textsuperscript{60}</td>
</tr>
<tr>
<td>GSN</td>
<td>Gelsolin</td>
<td>0.3</td>
<td>Apoptotic process\textsuperscript{69}</td>
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</table>
Chronic exposure to chewing tobacco alters the cellular proteome

Once we established that chronic exposure of chewing tobacco induces cellular transformation or leads to progression toward oncogenicity, we sought to study the molecular changes in the tobacco treated cells. We studied the alteration in the cellular proteome of OKF6/TERT1 and OKF6/TERT1-Tobacco cells using iTRAQ-based quantitative proteomic analysis. The experimental workflow followed is shown in Figure 2. LC-MS/MS analysis led to the identification of 3,636 proteins among which 174 proteins were overexpressed and 234 proteins were downregulated in the tobacco treated cells compared to the untreated cells (fold change cut-off ≥1.5). The complete list of proteins and their corresponding peptides with their iTRAQ ratios, m/z values and charge state is provided in Supplementary Tables S1 and S2. A partial list of differentially regulated proteins is provided in Table 1.

Chronic exposure to chewing tobacco leads to increased expression of SCD in oral keratinocytes

Our proteomics data revealed that SCD was upregulated 2.6-fold in cells chronically treated with chewing tobacco compared to the parental cells. A representative MS/MS spectrum is shown in Figure 3A. In agreement with mass spectrometry data, Western blot analysis revealed a significant increase in the expression of SCD in OKF6/TERT1-Tobacco cells compared to the parental OKF6/TERT1 cells (Fig. 3B). We evaluated the expression profile of SCD in a panel of HNSCC cell lines, JHU-O22, JHU-O28, JHU-O29, CAL27 and Fadu. As evident from Figure 3C, all HNSCC cell lines showed an increased expression of SCD compared to normal oral keratinocytes, OKF6/TERT1.

Inhibition of SCD decreases cellular proliferation

Having observed that SCD was overexpressed both in HNSCC cell lines and OKF6/TERT1-Tobacco cells, we examined the role of SCD in cellular proliferation. Cellular proliferation for the panel of HNSCC cell lines and OKF6/TERT1-Tobacco cells were studied after silencing endogenous expression of SCD using its specific siRNA. Western blot analysis post-transfection with SCD siRNA revealed a successful knockdown of SCD in OKF6/TERT1-Tobacco and HNSCC cell lines (Fig. 3C). We observed a decrease in cellular proliferation of the HNSCC cell lines and also in the OKF6/TERT1-Tobacco cells upon siRNA mediated silencing of SCD (Fig. 4A-E). To determine whether inhibition of SCD had any effect on cell survival, the OKF6/TERT1-Tobacco and HNSCC cells were treated with CAY10566, a specific inhibitor of SCD25 and cell proliferation

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**Figure 3.** Chronic exposure to chewing tobacco leads to increased expression of SCD in oral keratinocytes. (A) Representative MS/MS spectra of SCD. (B) Western blot analysis showed overexpression of SCD in OKF6/TERT1-Tobacco cells compared to OKF6/TERT1 cells. (C) OKF6/TERT1-Tobacco and a panel of HNSCC cell lines were transfected with SCD siRNA and Western blot was performed 48h post transfection using anti-SCD antibody. β-actin was used as a loading control.
assay was performed. Our results indicated that both the OKF6/TERT1-Tobacco cells and the panel of HNSCC cell lines showed a decrease in cell viability in presence of the inhibitor (Data not shown).

Inhibition of SCD reduces the colony formation ability of oral keratinocytes chronically exposed to chewing tobacco

Colony formation assay is often performed to understand the oncogenic potential or infinite growth potential of cells.26 Having observed that SCD plays an essential role in cellular proliferation, we next studied the effect of SCD ablation in the colony forming ability of the cells. HNSCC cell lines, including the OKF6/TERT1-Tobacco cells showed a decrease in their colony forming ability in the presence of SCD inhibitor, CAY10566 (Fig. 5A-B). Using an alternative strategy to suppress the expression of SCD in the HNSCC cell lines, we silenced SCD expression using its specific siRNA. In agreement to the results of SCD inhibitor, CAY10566, over the colony formation ability of the HNSCC cell lines, siRNA-mediated silencing of SCD in HNSCC cell lines resulted in a significant decrease in the colony formation ability of the cells (Fig. 5C-D). It is to be noted that, the OKF6/TERT1-Tobacco and JHU-O28 cells did not form discrete colonies. However, a significant decrease in cell population was observed both in the presence of SCD siRNA and CAY10566. Taken together, our results indicate that SCD plays an essential role in the early cellular changes induced by chewing tobacco that lead to malignancy.

Inhibition of SCD reduces the invasive property of cells exposed to chewing tobacco

Decreased colony formation potential is often associated with loss of invasion capabilities in cancer cells.27 Since invasion is a critical property for metastasis of tumor cells, we further investigated the in vitro invasive capabilities of the HNSCC cells using Matrigel coated transwell chambers. Silencing of SCD using siRNA in OKF6/TERT1-Tobacco and a panel of HNSCC cell lines decreased the invasive property of the cells (Fig. 6A-B). In concordance with the siRNA results, inhibition of SCD with its inhibitor, CAY10566, resulted in a significant decrease in the invasive property of both the HNSCC and OKF6/TERT1-Tobacco cells (Fig. 6C-D). Taken together, these results indicate that silencing of SCD decreases the invasive properties of HNSCC cells.

Discussion

Earlier studies have indicated that chronic exposure to cigarette smoke provides a better model in vitro than acute exposure to cigarette smoke.28 Number of cellular models have been developed and employed to understand the mechanisms of cellular transformation from normal to tumorigenic phenotype in response to chronic exposure to cigarette smoke.28-32 All the above studies have shown that chronic cigarette smoke exposure/
treatment induces selection of clones that have incorporated the molecular level changes necessary for cancer progression and resistance to apoptosis.

Smokeless tobacco or chewing tobacco is a known risk factor in the development of oral cancer. Although the tumor inducing property of chewing tobacco was proven in the year 1964, the molecular events that lead to tumor growth and progression upon its consumption are not known. It is important to understand the molecular mechanisms and pathobiology of oral cancer resulting from chewing tobacco as it differs significantly from cigarette smoking because of differences in composition. Chewing tobacco contains several compounds such as nicotine, tobacco-specific N-nitrosamines and polycyclic aromatic hydrocarbons which are known to be carcinogenic. As mentioned earlier tobacco demonstrates its carcinogenic effect through long-term (chronic) exposure and not acute. The goal of this study was to understand the total effects of chewing tobacco and not its individual components in inducing cellular transformation of normal oral keratinocytes upon chronic exposure. Toward this end, we developed a cellular model that mimics the in vivo system of chronic habit of chewing tobacco.

We demonstrate that chronic exposure to chewing tobacco induces molecular changes in a cellular system that is a pre-requisite for progression to malignancy. This is evident by the increased cellular proliferation and invasion ability of the tobacco treated cells. Apoptotic signaling by Bax and Bak can be sequestered by Bcl-xL, leading to cellular survival. Our results indicated an increase in the expression of Bcl-xL and Bcl-2 in the chewing tobacco treated cells compared to the parental cells. Bcl-2 has been shown to be overexpressed in chewing tobacco-induced oral cancer. In addition, Bcl-xL has been found to be upregulated in other cancers and is considered to be a marker for increased tumorigenesis. An increase in the Bcl-xL/Bax ratio provides resistance to DNA damage and aids in survival of cancer cells. The increase in the Bcl-xL/Bax ratio in the tobacco treated cells probably leads to an increase in cellular survival due to selective pressure of the tumor microenvironment which enables them to acquire oncogenic property. This was supported by mass spectrometry-based analysis of the cell line pair (OKF6/TERT1-Parental and OKF6/TERT1-Tobacco). A wide array of proteins known to play an essential role in increased cellular proliferation were found to be overexpressed in the cells chronically treated with tobacco. These proteins included TIMP metallopeptidase inhibitor 1 (TIMP1) (2.7-fold), the antigen identified by monoclonal antibody Ki-67 (MKI67) (2.8-fold) and filamin B (FLNB) (2.0-fold). These observations provide a possible explanation for enhanced cellular proliferation in the tobacco...
treated cells. We also observed a decrease/loss of expression of multiple tumor suppressor genes. This includes, programmed cell death protein 4 (PDCD4) (0.3-fold) and S100 calcium binding protein A2 (S100A2) (0.4-fold).40,41

Our proteomics data also showed an increased expression of proteins such as epidermal growth factor receptor pathway substrate 8 (EPS8) (2.0-fold), S100 calcium binding protein A14 (S100A14) (1.9-fold) and heat shock 70kDa protein 5 (HSPA5) (1.6-fold), which are known to be aberrantly expressed in head and neck cancer.42-44 This data supports our hypothesis that chronic exposure to chewing tobacco induces malignancy and potentiates the induction of head and neck carcinoma. Apart from the known proteins in literature, we also identified few novel proteins (enhancer of rudimentary homolog (ERH) (7.1-fold) and teneurin transmembrane protein 2 (TENM2) (0.3-fold), whose role in HNSCC remains to be elucidated, which is beyond the scope of this manuscript.

Our observation that SCD is overexpressed in HNSCC is supported by studies in other cancer types.17,21,45 SCD is known to induce epithelial to mesenchymal transition and cancer progression.46 SCD mediates its effects through activation of various signaling events such as activation of PI3K/AKT pathway, Wnt/β-catenin and through the activation of MAP kinases (MAPK1/3).45-47 Here, we show a direct effect of chewing tobacco on normal oral cells and provide evidence that increased expression of SCD in presence of chewing tobacco leads to oncogenic transformation of oral cells. We further demonstrate that inhibition of SCD results in loss of oncogenic and metastatic potential of head and neck cancer cells. Taken together, our results indicate that SCD plays an essential role in HNSCC progression and can serve as a potential therapeutic target in head and neck cancer. Our current study does not rule out the role of other proteins in the progression of HNSCC. In addition, the precise mechanisms of how SCD acts as a therapeutic agent in HNSCC have not yet been clearly defined. However, a detailed understanding of the role of SCD in HNSCC progression is needed and requires both in vivo and in vitro models. In this study, we report the linkage of overexpression of SCD to chewing tobacco mediated oncogenic transformation in oral cells that has not been previously reported.

**Materials and Methods**

**Preparation of chewing tobacco extract**

Chewing tobacco was procured from local supplier. 10 g of tobacco was finely powdered and homogenized in 100 ml of 1X phosphate buffer saline (PBS). The mixture was stirred at 37°C for 24h, followed by centrifugation at 2000 g for 15 min. The supernatant was collected and sterilized using 0.22 µm filter.12 This was considered as 100% extract of chewing tobacco. The
chewing tobacco extract was aliquoted and stored at −80°C until further use.

Cell culture

Normal human oral keratinocytes, OKF6/TERT1, were a generous gift from Dr. James Rheinwald (Brigham and Women’s Hospital, Boston, MA). OKF6/TERT1 were cultured and maintained in keratinocyte serum free medium (KSF) supplemented with bovine pituitary extract (25 µg/ml), epidermal growth factor (EGF) (0.2 ng/ml), 1% penicillin/streptomycin and CaCl₂ (0.4 mM). Fadu and CAL27 cells were procured from American Type Culture Collection (ATCC, Manassas, VA). JHU-O22, JHU-O28, JHU-O29 and Fadu cells were grown in RPMI media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin mixture. CAL27 cells were grown and maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin. All cells were grown at 37°C in a humidified 5% CO₂ incubator.

Treatment of OKF6/TERT1 cells with chewing tobacco

OKF6/TERT1 cells were chronically treated with chewing tobacco extract at 1% concentration for 6 months. Henceforth, OKF6/TERT1 cells chronically treated with chewing tobacco extract will be termed as “OKF6/TERT1-Tobacco” and the parental cells will be referred to as “OKF6/TERT1.” Both these cells were grown at 37°C in a humidified 5% CO₂ incubator.

siRNA transfection

ON-TARGETplus SMARTpool control siRNA (catalog # D-001810–10–05, Invitrogen, Carlsbad, CA) and SCD siRNA (catalog # L-005061–00–0005, Invitrogen, Carlsbad, CA) were purchased from Dharmacon (Lafayette, CO). Cells were transiently transfected with scrambled or SCD siRNA using Lipofectamine RNAiMAX reagent (catalog # 13778075, Invitrogen, Carlsbad, CA) as described previously.30

Cell proliferation assays

OKF6/TERT1-Tobacco cells and HNSCC cell lines were seeded at a density of 12 × 10³ cells per well in 12-well plates. The cells were transfected with either SCD siRNA or control siRNA. The cellular proliferation was monitored for 3 d post-transfection where the cells were counted using trypan blue exclusion method. All assays were carried out in triplicate.

Sample preparation

OKF6/TERT1 and OKF6/TERT1-Tobacco cells were grown to 80% confluence. The cells were starved for 12h. Then, the cells were washed with 1X PBS and harvested in 1X ice-cold PBS. Cells were lysed in 0.5% SDS buffer followed by sonication (Branson Sonifier, Danbury, CT) and centrifugation. The cleared supernatant was used for protein estimation using the bicinchoninic acid assay (BCA).48

In-solution digestion and iTRAQ labeling

In-solution trypsin digestion and iTRAQ labeling of samples from both conditions was carried out as described previously.49 Equal amounts of cell lysate (200 µg) from both OKF6/TERT1 and OKF6/TERT1-Tobacco cells were reduced with (tris(2-carboxyethyl) phosphine (TCEP)) at 60°C for 1h. This was followed by alkylation with cysteine blocking reagent, methyl methanethiosulfonate (MMTS) for 10 min at room temperature. The samples were then subjected to trypsin digestion at 37°C for 12–16 h using sequencing grade trypsin (catalog # V5111, Promega, Madison, WI) at enzyme to substrate ratio of 1:20. Following trypsin digestion, the peptides were labeled with iTRAQ reagents as follows, peptides from OKF6/TERT1 cells were labeled with reagents containing the reporter ions of m/Z 114 and 115 and those from OKF6/TERT1-Tobacco cells were labeled with reagents containing reporter ions of m/Z 116 and 117. The samples from both the conditions were pooled and subjected to fractionation.

Strong cation exchange chromatography (SCX)

SCX fractionation of the iTRAQ labeled peptides was performed as described previously.30 Pooled sample was diluted to 1ml with solvent A (10mM KH₂PO₄, 25% (v/v) ACN, pH 2.7). The pH of sample was adjusted to 2.7 using ortho-phosphoric acid. The peptides were loaded on PolySULFOETHYL A column (PolyLC, Columbia, MD) (5 µm, 200×2.1mm) using an Agilent 1290 Infinity series HPLC system (Agilent Technologies, Santa Clara, CA). Peptides were fractionated using a 50 min gradient from 0% to 40% solvent B (350 mM KCl in solvent A). A total of 96 fractions were collected and further pooled into 23 fractions based on chromatographic peaks. The pooled fractions were dried and desalted using C₁₈ StageTips and stored at −20°C till further analysis.

LC-MS/MS analysis

LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) interfaced with Proxeon Easy nLC system (Thermo Scientific, Bremen, Germany) was used for proteomic analysis. Enrichment of the peptides was carried on a trap column (75 µm × 2cm) packed in-house using C₁₈ material (Magic C₁₈AQ, 5 µm, 100A, Michrom Biosciences Inc.) with a flow rate of 3 µl/min using solvent A (0.1% formic acid) and resolved on an analytical column (75 µm × 10 cm, Magic C₁₈AQ, 3 µm, 100A, Michrom Biosciences Inc.) at a flow rate of 350 nL/min using a linear gradient of 7-30% solvent B (95% acetonitrile, 0.1% formic acid) over 70 min. MS and MS/MS scans were acquired with a mass resolution of 60,000 and 15,000 at 400 m/z using the Orbitrap mass analyzer. Precursor MS scan was set to m/z 350–1,800. In each duty cycle 20 most intense monoisotopic precursors were selected for MS/MS fragmentation using higher-energy collision dissociation (HCD) mode at 41% normalized collision energy. Isolation width was set to 1.9 m/z. Singly charged and unassigned charge precursor ions were rejected. Dynamic exclusion setting was enabled and acquired ions were excluded for 45s. The automatic gain control for full MS and MS/MS were set to 1 × 10⁶ and 5 × 10⁴ ions, respectively. The maximum ion injection time was set to 100 ms for MS and 250 ms for MS/MS scans. The lock mass option was enabled using polydimethylcyclotiosiloxane ions (m/z, 445.120025) for internal calibration.
Data analysis

The raw data obtained was searched using Sequest and Mascot (version 2.2.0, Matrix Science, London, UK) search algorithms through Proteome Discoverer (version 1.4.0.288, Thermo Scientific, Bremen, Germany) suite against NCBI RefSeq human protein database (version 65 containing 34,453 protein sequences and known contaminants). The search parameters included tryptic as proteolytic enzyme with 1 missed cleavage and oxidation of methionine as variable modification. The static modifications included alkylation (methylthio) at cysteine and iTRAQ modification at N-terminus of the peptide and lysine. Mass tolerances of precursor and fragment ions were set to 20 ppm and 0.1 Da, respectively. False discovery rate of 1% was considered to report identification. The average of reporter ion intensities from technical replicates was used for iTRAQ quantitation. Quantitation of identified peptide was normalized on protein median.

Western blotting

Cells were grown to 80% confluency and the proteins were harvested in RIPA lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton-X-100, 0.1% SDS containing protease and phosphatase inhibitor cocktails) and sonicated. Western blot analysis was carried out as described previously.30 Briefly, 30 μg of the cell lysate was resolved by SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in PBS-T and incubated overnight with monoclonal mouse anti-SCD antibody (catalog # ab19862, Abcam, Cambridge, MA). Anti-mouse IgG bound to the HRP conjugate was used as a secondary antibody. Proteins on the membrane were visualized using enhanced chemiluminescence detection kit as per manufacturer’s instructions. β-Actin was used as loading control for all Western blots.

Cell invasion assays

Invasion assay was performed in a transwell system (BD Biosciences, San Jose, CA) with Matrigel coated filters and cellular invasion was evaluated after 48h. Briefly, invasiveness of the cells was assayed in the membrane invasion culture system using polyethylene terephthalate (PET) membrane (8-m pore size) (Catalog # 353097, BD Biosciences, San Jose, CA) in the upper compartment of a transwell coated with Matrigel (Catalog # 354234, BD Biosciences, San Jose, CA). The cells were seeded at 2.0 × 10^4 cells in 500 μl of media on the Matrigel-coated PET membrane in the upper compartment. The lower compartment was filled with complete growth media and the plates were incubated at 37°C for 48 h. At the end of the incubation time, the upper surface of the membrane was wiped with a cotton-tip applicator to remove nonmigratory cells. Cells that migrated to bottom side of membrane were fixed and stained using 4% methylene blue. Each measurement was performed in duplicate. All experiments were repeated thrice.

Colonies formation assays

OKF6/TERT1-Tobacco and the HNSCC cell lines were transfected with either SCD siRNA or control siRNA. Post-transfection 3×10^3 cells were seeded into 6-well plates with complete media. Cell colonies were allowed to grow for 10–14 days, before the colonies were fixed with methanol and stained with 4% methylene blue. The number of colonies per well was counted. Similarly, colony formation ability of the OKF6/TERT1-Tobacco and HNSCC cells was monitored in presence of SCD inhibitor, CAY10566 (3-[4-(2-chloro-5-fluorophenoxy)-1-piperidinyl]-6-(5-methyl-1,3,4-oxadiazol-2-yl)-pyridazine) (Catalog # CAY10566, Cayman Chemicals Ann Arbor, USA). All experiments were performed in triplicate.

Data availability

The mass spectrometry data files generated in this study can be accessed freely available at ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE public data repository.51 The data can be downloaded using dataset identifier PXD001554. Alternatively, the peptides and their associated MS/MS spectra can be downloaded from Human Proteinpedia at http://www.humanproteinpedia.org/data_display?exp_id=00804.52

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.