Article:
Patil, S, Patel, K, Advani, J et al. (9 more authors) (2019) Multiomic analysis of oral keratinocytes chronically exposed to shisha. Journal of Oral Pathology and Medicine, 48 (4). pp. 284-289. ISSN 0904-2512

https://doi.org/10.1111/jop.12828

DR HARSHA GOWDA (Orcid ID : 0000-0002-4118-6855)

Article type : Original Article

Multiomic analysis of oral keratinocytes chronically exposed to shisha

Shankargouda Patil[1,2#], Krishna Patel[3#], Jayshree Advani[3#], Tejaswini Subbannayya[3], Pavithra Rajagopalan[3], Niraj Babu[3,4], Hitendra Solanki[3], Shilpa Bhandi[5], David Sidransky[6], Aditi Chatterjee[3,4], Harsha Gowda[3,4]*, Marco Ferrari[1,7]*

[1]Department of Medical Biotechnologies, School of Dental Medicine, University of Siena, Italy

[2]Department of Maxillofacial Surgery and Diagnostic Sciences, Division of Oral Pathology, College of Dentistry, Jazan University, Jazan, Saudi Arabia

[3]Institute of Bioinformatics, International Technology Park, Bangalore 560 066, India

[4]Manipal Academy of Higher Education (MAHE), Manipal-576104, Karnataka, India

[5]Department of Restorative Dental Sciences, Division of Operative Dentistry, College of Dentistry, Jazan University, Jazan, Saudi Arabia

[6]Department of Otolaryngology-Head and Neck Surgery, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA

[7]Department of Restorative Dentistry, School of Dentistry, University of Leeds, UK

[#]Authors contributed equally

Corresponding authors

Harsha Gowda

Institute of Bioinformatics, 7th floor, Discoverer building, International Tech Park, Bangalore 560 066, India.

E-mail: harsha@ibioinformatics.org, Telephone: 918028416140, Fax: 918028416132

Marco Ferrari

Department of Prosthodontics & Dental Materials and Dean, School of Dental Medicine, University of Siena, Italy

and

Department of Restorative Dentistry, School of Dentistry University of Leeds, UK

E-mail: ferrarm@gmail.com, Telephone: 393403277096, Fax: 393403277097

Running title

Oral cells chronically exposed to shisha

Keywords

Hookah, Next generation sequencing, Oral health, Waterpipe smoking, Carcinogenesis

Conflict of interest

The authors report no conflict of interest.

Abstract

Background: Tobacco is smoked in different form including cigarettes and water pipes. One popular form of water pipe smoking especially in Middle Eastern countries is shisha smoking. Shisha has been associated with various diseases including oral cancer. However, genomic alterations and gene expression changes associated with chronic shisha exposure have not been previously investigated.

Objectives: Whole exome sequencing and gene expression profiling of immortalized human oral keratinocytes (OKF6/TERT1) cells chronically treated with 0.5% shisha extract for a period of 8 months was undertaken to characterize molecular alterations associated with shisha exposure.

Methods: Genomic DNA and RNA were extracted and preprocessed as per manufacturer's instruction and subjected to whole exome and transcriptome sequencing using Illumina HiSeq2500 platform. Exome was analyzed using GATK pipeline whereas RNA-Seq data was analyzed using HiSat2 and HTSeq along with DESeq to elucidate differentially expressed genes.

Results: Whole exome sequence analysis led to identification of 521 somatic missense variants corresponding to 389 genes RNASeq data revealed 247 differentially expressed genes (≥2 fold, p value<0.01) in shisha treated cells compared to parental cells. Pathway analysis of differentially expressed genes revealed that interferon signaling pathway was significantly affected. We predict activation of MAPK1 pathway which is known to play a key role in oral cancer. We also observed allele specific expression of mutant LIMA1 based on RNA-Seq dataset.

Conclusion: Our findings provide insights into genomic alterations and gene expression pattern associated with oral keratinocytes chronically exposed to shisha.

1. Introduction

Tobacco smoking is the leading cause of morbidity and mortality worldwide. Each year more than 7 million people die due to regular use of tobacco [1]. Shisha, also known as water pipe smoking, hookah and 'hubble bubble' is a popular way of smoking tobacco which is more prevalent in the Middle East, India, North Africa and Southeast Asia amongst other countries [2]. A recent study has stated that use of shisha increases chances of oral squamous cell carcinoma (OSCC) by 4 fold whereas smoking is attributed to increase it by 1.6 fold [3]. Oral cancer is sixth most common cancer in the world [4]. Despite these statistics, molecular alterations associated with oral cancer due to shisha smoking have not been extensively investigated [5].

A recent study on young shisha smokers and non-smokers has demonstrated that shisha smoking is associated with increased cough and sputum, increased levels of pulmonary capillary-derived endothelial microparticles and marked transcriptional changes in alveolar macrophages and in small airway epithelium [6]. A further study on small airway epithelium highlights association of shisha smoking with epigenetic changes and related transcriptional modifications that impact a number of genes and pathways previously reported to be associated with cigarette smoking [7]. Studies using mouse models have demonstrated that exposure to shisha smoking for 1 month increased airway resistance, inflammation and oxidative stress [8]. A comparative study on exposure to tobacco-specific nitrosamines (NNAL) in shisha smokers, cigarette smokers and non-smokers has shown that regular shisha smokers are exposed to about 5-10 times greater quantities of nitrosamines than non-smokers which is equivalent to pack-a-day cigarette smokers [9].

Risk associations and pathogenesis of oral cancer is well documented [10, 11] however, it is not extensively studied in shisha smokers. We developed a cell line model using immortalized normal oral keratinocytes by chronically treating them with shisha extract for a period of 8 months. We have previously investigated cellular models using cigarette smoke and chewing tobacco to understand molecular alterations associated with different forms of tobacco [12, 13].

In this study, we investigated mutational spectrum and dysregulation of gene expression pattern in immortalized normal human oral keratinocytes (OKF6/TERT1) chronically treated with shisha extract. To our knowledge, this is the first whole exome sequencing and transcriptome profiling study to delineate genomic alterations and gene expression changes in oral cells due to chronic shisha exposure.

2. Methods

2.1 Cell culture

Immortalized normal human oral keratinocyte (OKF6/TERT1) cell line was a kind gift from Dr. James Rheinwald at Brigham and Women's Hospital in Boston, MA [14]. OKF6/TERT1 cells were cultured using keratinocyte serum-free media (KSFM) (Life Technologies, Grand Island, NY) which was supplemented with bovine pituitary extract (25 mg/ml), calcium chloride (0.4 mM), epidermal growth factor (0.2 ng/ml) and 1% penicillin/streptomycin solution as described previously [15]. Shisha extract was prepared by following a similar method described previously [16]. 5 gm of commercially available shisha was homogenized in 50 ml of phosphate buffered saline (PBS). The mixture was stirred overnight in a shaker incubator maintained at 37ºC. This was followed by centrifuging extract at 12,000 rpm for 20 min and supernatant was filtered to remove debris. The extract was sterilized using a 0.22 μm filter and stored at -80ºC until further use. To study the effects of shisha on oral keratinocytes, cells were chronically treated with 0.5% shisha extract (hereafter referred as OKF6/TERT1-Shisha) maintained in humidified chamber with 5% CO2 at 37ºC for a period of 8 months. A typical session of shisha smoking ranges between 30 mins to a few hours, and utilizes about 15g of shisha [17]. Since it is not feasible to replicate these conditions in-vitro, the concentration of shisha extract used was standardized to achieve maximum exposure with minimum cell death. OKF6/TERT1-Parental and OKF6/TERT1-Shisha cells were grown to 80% confluence. Subsequently, the growth media was removed and cells were washed thrice using phosphate buffered saline (1X PBS). Cells were harvested in PBS and genomic DNA and RNA was extracted from OKF6/TERT1–Parental and OKF6/TERT1-Shisha cells.

2.2 Whole exome sequence analysis

Library preparation was done using Agilent SureSelectXT Human All Exon V5 kit as per manufacturer's instructions. Samples were multiplexed and then subjected to sequencing on an Illumina HiSeq 2500 platform. Raw reads were obtained in FASTQ format from Illumina HiSeq 2500 sequencing platform. These reads were assessed for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Terminal bases with Phred score ≤ 20 or adapter sequences were trimmed off and clean reads were aligned against human reference genome HG19 (GRCh37) using BWA-MEM (version 0.7.10) with default parameters. Binary alignment map (BAM) files were further processed to mark duplicate reads that can be potential PCR artefacts using MarkDuplicates of Picard tools (version 2.0.1). BAM file with duplicate reads marked were subjected to indel realignment and base recalibration using IndelRealigner and BaseRecalibrator respectively of GATK4 tool suite (the Genome Analysis Toolkit, Broad Institute). Pre-processed BAM files were converted to pileup format using mpileup module of Samtools. mpileup files were further analyzed using VarScan2 (http://varscan.sourceforge.net/) to elucidate somatic variants with allele supporting reads ≥ 8 in tumor, allele fraction of 0.2 and somatic p-value ≤ 0.01. Single nucleotide variants (SNVs) were annotated using Annovar.

2.3 Whole transcriptome sequence analysis

RNA sequencing libraries were constructed according to the manufacturer's instructions from total RNA extracted using Illumina TruSeq RNA Preparation kit. The libraries so obtained were then multiplexed and subjected to sequencing (150 bp paired-end reads) on Illumina HiSeq 2500. Raw reads were obtained in Fastq format and were assessed for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Terminal low quality bases and adaptor sequences were trimmed off using Fqtrim (version 0.94) retaining 70 bases as minimum read length cut-off. Clean reads were aligned against reference genome HG38 (GRCh38) obtained from Gencode (version 26) using HiSat2 with default parameters (https://ccb.jhu.edu/software/fqtrim/, version 2.0.1). BAM files obtained from HiSat2 were further processed with HTSeq (https://htseq.readthedocs.io, version 0.6.1) to quantify raw read counts in gene mode for OKF6/TERT1–Parental and

OKF6/TERT1-Shisha datasets. R package DESeq (https://www.huber.embl.de/users/anders/DESeq/) was subsequently employed to ascertain list of differentially expressed genes (DEGs) with fold change ≥ 2 and p-value ≤ 0.01. BAM files were also used to quantify expression of each gene in fragments per kilobase of transcript per million mapped reads (FPKM) unit using Stringtie (https://ccb.jhu.edu/software/stringtie/, version 1.2.0)

2.4 Integration of whole exome and RNA-Seq dataset

To assess the allele specific expression of somatic SNVs, BAM files of OKF6/TERT1-Shisha RNA-Seq dataset obtained from HiSat2 was preprocessed using MarkDuplicates of Picard tools (version 2.0.1), followed by SplitNCigarReads, IndelRealigner and BaseRecalibrator of GATK4 tool suite. Base recalibrated RNA-Seq BAM file was further processed using ASECount of GATK4 tool suite with default parameters.

Somatic mutation signature was plotted using R package SomaticSignatures (http://bioconductor.org/packages/release/bioc/html/SomaticSignatures.html). Functional annotation of DEGs was carried out using FunRich (www.funrich.org) and Pathway enrichment analysis using QIAGEN's Ingenuity Pathway Analysis (IPA®, http://www.qiagen.com/ingenuity).

3. Results

Long term smoking has been previously associated with irreversible changes in cells of squamous origin [18]. We assessed phenotypic alteration in OKF6/TERT1-Shisha cells compared to OKF6/TERT1-Parental cell. We observed increased proliferation rates, invasiveness and cell scattering phenotype in OKF6/TERT1 cells chronically treated with 0.5% shisha extract for 8 months (manuscript under review). However, systematic studies on shisha smoking and its effects on oral cells is lacking. We therefore employed exome sequencing and transcriptome profiling to elucidate the effect of chronic shisha exposure on the exome and consequently the transcriptome of normal oral keratinocytes.

3.1 Whole exome sequence analysis

Whole exome sequencing of OKF6/TERT1-Parental and OKF6/TERT1-Shisha cells resulted in ~108 million reads in OKF6/TERT1–Parental and ~60 million reads in OKF6/TERT1-Shisha with 100 bp and 150 bp respectively. Read quality was assessed using FastQC and we observed 97.04% reads with Phred score ≥20 in OKF6/TERT1-Parental and 96.20% in OKF6/TERT1-Shisha. Target coverage of 99.84% in OKF6/TERT1–Parental and 97.72% in OKF6/TERT1–Shisha was obtained with ≥ 10X depth in whole exome datasets.

Somatic variants analysis led to identification of 521 SNVs in OKF6/TERT1-Shisha cells. We observed C>A transition which is associated with smoking history and C>T transversion which is known to be associated with various cancers **(Figure 1)**. Among 521 SNVs, 303 (~59%) were non-synonymous and 5 were splice site variants. We observed somatic missense SNVs in genes including SASH1, FLNC, and ADCY8 that were predicted to be deleterious by ≥ 3 prediction algorithms **(Supplementary Table 1)**. These three genes are known to be recurrently mutated in head and neck squamous cell carcinoma [19]**.**

3.2 Transcriptome analysis

Poly-A tail enriched transcriptome profiling resulted in ~106 million reads and ~82 million reads in OKF6/TERT1–Parental and OKF6/TERT1-Shisha cells, respectively. High quality reads were filtered using Fqtrim retaining bases with Phred score ≥30 and minimum read length of 70 resulting in ~99 million reads and ~76 million reads in OKF6/TERT1–Parental and OKF6/TERT1-Shisha transcriptome datasets, respectively. For differential expression analysis, genes identified with ≥30 reads in both OKF6/TERT1–Parental and OKF6/TERT1-Shisha datasets i.e. 11,921 genes were interrogated using DESeq. This analysis revealed 247 genes with fold change ≥ 2 and p-value ≤ 0.01 of which 101 were overexpressed and 146 were downregulated **(Supplementary Table 2)**.

Gene Ontology enrichment using FunRich revealed enrichment of immune response (15.4%) and cytoplasm (66.7%) in downregulated genes whereas cell growth and/or maintenance (14.3%) and exosomes (25%) were enriched in overexpressed genes. Genes in down regulated cohort mainly contributed to immune response and were classified under molecular classes of cytokines (ISG15, 39.9 fold; CSF2, 3.9 fold), complement proteins (C1S, 10.7 fold; C1R, 8.3 fold), and GTPases (MX2, 104.3 fold; SAMHD1, 12.3 fold); whereas over expressed genes enriched cell growth and/or maintenance processes and were categorized under cytoskeletal protein (TUBGCP3, 2.6 fold; PLEKHH3, 2.5 fold; PPL, 4.18 fold; KRT16, 3.8 fold; KRT17, 3.7 fold), cell junction protein (CLDN1, 4.3 fold) and extracellular matrix protein (COL5A2, 5.5 fold) among other dysregulated genes as stratified by Human Protein Reference Database (HPRD) [20].

3.3 Integrative analysis of whole exome and RNA-Seq

Integrative analysis of whole exome and transcriptome profiles would allow us to investigate effect of somatic variants on expression levels of their corresponding genes. Median FPKM value of genes identified with missense variant in OKF6/TERT1-Shisha is 1.09 whereas in OKF6/TERT1-Parental is 2.69. Similarly, median FPKM value of genes identified with silent variants in OKF6/TERT1-Shisha is 3.12 and 4.49 in OKF6/TERT1-Parrental cells.

We also evaluated RNA-Seq data for allele specific expression of somatic missense SNVs. We observe that gene LIM domain and actin binding 1 (LIMA1) which is present on chr12, harbors a missense SNV g.T50571445A which leads to amino acid change from glutamine to valine. This SNV has alternate read support of 9 with allele frequency 75% and is predicted to be deleterious by SIFT and PolyPhen in WXS dataset. In RNA-Seq dataset, there are 278 reads supporting this genomic position and 123 reads out of 278 supported mutant allele resulting in allele frequency of 44.24%.

3.4 Pathway analysis using Ingenuity Pathway Analysis (IPA)

To evaluate altered pathways in OKF6/TERT1-Shisha cells, bioinformatics analysis was employed on DEGs using IPA and we observe Interferon signaling pathway was significantly enriched (p-value < 0.01) **(Supplementary Table 3)**. Multiple genes associated with interferon signaling such as integral membrane protein (IFITM2, 3.84-fold; IFITM3, 3.7-fold), transcription factor (STAT1, 9-fold; STAT2, 3.12-fold), cytokine (ISG15, 50-fold), GTPase (MX1, 100-fold), and transcription regulatory protein (IRF1, 3.84-fold) were seen to be downregulated in OKF6/TERT1-Shisha cells compared to parental cells. In conjunction with this analysis, upstream regulator network analysis predicted inhibition of IFNA2 (z-score: -5.3), interferon alpha (z-score: -3.53), and IFNA1/IFNA13 (z-score: -2.17) with pvalue < 0.01 **(Figure 2A)**.

3.5 Comparison of OKF6/TERT1-Smoke and OKF6/TERT1-Shisha

Previous study by our group elucidated the molecular alterations in oral cells upon chronic cigarette smoke exposure [12]. In that study, OKF6/TERT1-Parental cells were chronically treated with cigarette smoke condensate (CSC) for 8 months and molecular alterations associated with treated cells were characterized by carrying out whole exome sequencing and protein expression profiling. In this study, we characterized molecular alterations associated with OKF6/TERT1-Shisha by carrying our whole exome sequencing and transcriptome profiling. We compared genomic anomalies identified using WXS of OKF6/TERT1-Shisha with WXS of OKF6/TERT1-Smoke. We observed higher frequency of C>T transversions associated with exposure to two forms of tobacco. Signature 2 characterized by high C>T transversions is known to be associated with various cancers [21]. We did not observe any commonalities among genomic anomalies, however pathway analysis using IPA predicted activation of mitogen-activated protein kinase 1 (MAPK1) pathway (z-score ≥ 2) **(Figure 2B)**. MAPK1 network in OKF6/TERT1-Smoke shared 98% of the differentially expressed proteins with DEGs in OKF6/TERT1-Shisha suggesting common downstream effects resulting in cellular transformation in both smoke and shisha exposed oral cells.

4. Discussion

The perception of minimal detrimental effects of shisha smoke has contributed to dramatic increase in prevalence of shisha smoking. There are numerous epidemiological studies which have associated increased risk of esophageal, head and neck [22] and lung cancer [22-24] development with shisha smoking. However, laboratory studies to yield mechanistic insights into shisha mediated molecular alterations has not been carried out. To the best of our knowledge, this is the first in vitro cell line-based study to demonstrate the effect of chronic shisha extract exposure on non-neoplastic immortalized oral cells.

Shisha extract exposure resulted in a number of genomic aberrations previously associated with tumorigenesis. Reported mutation burden in oral squamous cell carcinoma is between 2 - 12 mutations per MB [10, 11]. We observed 10 mutations per MB in cells treated with shisha extract with similar mutation spectrum as reported in whole exome sequence analysis study of 15 OSCC patients with history of Shamma exposure [25]. In addition, missense variant(s) were identified in SAM and SH3 domain containing 1 (SASH1), Filamin C (FLNC), and Adenylate Cyclase 8 (ADCY8) with >30% allele frequency supported by at least 10 reads and predicted to be deleterious by $\geq$ 3 predictors. SASH1 is potential tumor suppressor gene functionally characterized in lung cancer and its induction is reported to inhibit cell proliferation and promote cellular apoptosis [26]. FLNC is an actin filament crosslink protein which is recurrently mutated in 11-36% of lung cancer [27]. Adenylate Cyclase 8 plays crucial role in calcium signaling pathway and is involved in apoptosis [28].

Pathway analysis with differentially expressed genes highlighted interferon signaling as one of the most enriched pathways in OKF6/TERT1-Shisha cells with 38.9% overlap with back end curated pathway. In addition, upstream regulator analysis delineated a network of genes downstream of serine/threonine kinase mitogen-activated protein kinase 1 (MAPK1) that were significantly downregulated in immortalized oral cells treated with shisha extract. These downregulated genes were enriched in biological processes primarily involved in cell immune response and cell-cell communication. Analysis also revealed 68.6% enrichment of dysregulated genes predicted to be

downstream of transcription factor IRF1. In a recent study, MAPK inhibition is reported to attenuate mechanisms that reduce IRF1 expression in pulmonary A549 cells [29].Genes regulated by IRF1 are also key players in host defense indicating enrichment of immune response associated proteins. MAPK pathway is known to play a role in promoting cell proliferation, inhibiting apoptosis, promoting tumor angiogenesis and inducing invasion and metastasis in oral cancer [30]. We have previously observed marked overexpression and activation of MAPK1 in OKF6/TERT1 cells chronically exposed to cigarette smoke [12]. Despite distinct genomic anomalies in OKF6/TERT1 cells chronically treated with cigarette smoke and shisha extract, interferon-signaling cascade was a commonly affected pathway in both models.

We observed marginal decrease in expression of genes which harbored somatic missense variants in OKF6/TERT1-Shisha cells compared to parental cells. We observed 10 genes that showed abundant expression (≥30 reads) in shisha treated cells with no detectable expression in untreated parental cells. In addition, we observed 49 genes that were abundantly expressed in parental cells but no detectable expression in shisha treated cells. We also observed expression of mutant LIMA1 with 123 read support and 44.24% allele frequency.

In conclusion, we have catalogued various genomic and transcriptomic alterations previously categorized in various cancers which highlights possible detrimental effects of shisha extract exposure. Further validation of these genetic changes is warranted in patients with shisha smoking history to establish their clinical relevance and their role in tumorigenesis in oral cells.

5. Acknowledgment and funding

We thank the Department of Biotechnology (DBT), Government of India for research support to the Institute of Bioinformatics. Krishna Patel and Niraj Babu are the recipients of Senior Research Fellowship from the Council for Scientific and Industrial Research (CSIR), Government of India.

6. Approved by ethics committee/institute

Not applicable

7. Conflict of interest

The authors report no conflict of interest.

8. References

1. WHO report on the global tobacco epidemic 2017. WHO report on the global tobacco epidemic. 2017.

2. Meo SA, Bashir S, Almubarak Z, Alsubaie Y, Almutawa H. Shisha smoking: impact on cognitive functions impairments in healthy adults. Eur Rev Med Pharmacol Sci. Nov 2017;21(22):5217-5222.

3. Alharbi F, Quadri MFA. Individual and Integrated Effects of Potential Risk Factors for Oral Squamous Cell Carcinoma: A Hospital-Based Case-Control Study in Jazan, Saudi Arabia. Asian Pac J Cancer Prev. Mar 27 2018;19(3):791-796.

4. Rodriguez T, Altieri A, Chatenoud L, et al. Risk factors for oral and pharyngeal cancer in young adults. Oral Oncol. Feb 2004;40(2):207-213.

5. Awan KH, Siddiqi K, Patil S, Hussain QA. Assessing the Effect of Waterpipe Smoking on Cancer Outcome - a Systematic Review of Current Evidence. Asian Pac J Cancer Prev. Feb 1 2017;18(2):495-502.

6. Strulovici-Barel Y, Shaykhiev R, Salit J, et al. Pulmonary Abnormalities in Young, Light-Use Waterpipe (Hookah) Smokers. Am J Respir Crit Care Med. Sep 1 2016;194(5):587-595.

7. Walters MS, Salit J, Ju JH, et al. Waterpipe smoking induces epigenetic changes in the small airway epithelium. PLoS One. 2017;12(3):e0171112.

8. Nemmar A, Raza H, Yuvaraju P, et al. Nose-only water-pipe smoking effects on airway resistance, inflammation, and oxidative stress in mice. J Appl Physiol (1985). Nov 1 2013;115(9):1316-1323.

9. Al Ali R, Rastam S, Ibrahim I, et al. A comparative study of systemic carcinogen exposure in waterpipe smokers, cigarette smokers and non-smokers. Tob Control. Mar 2015;24(2):125-127.

10. Mutational landscape of gingivo-buccal oral squamous cell carcinoma reveals new recurrently-mutated genes and molecular subgroups. Nat Commun. 2013;4:2873.

11. Upadhyay P, Gardi N, Desai S, et al. Genomic characterization of tobacco/nut chewing HPV-negative early stage tongue tumors identify MMP10 asa candidate to predict metastases. Oral Oncol. Oct 2017;73:56-64.

12. Rajagopalan P, Patel K, Jain AP, et al. Molecular alterations associated with chronic exposure to cigarette smoke and chewing tobacco in normal oral keratinocytes. Cancer Biol Ther. May 3 2018:1-13.

13. Nanjappa V, Renuse S, Sathe GJ, et al. Chronic exposure to chewing tobacco selects for overexpression of stearoyl-CoA desaturase in normal oral keratinocytes. Cancer Biol Ther. 2015;16(11):1593-1603.

14. Dickson MA, Hahn WC, Ino Y, et al. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. Mol Cell Biol. Feb 2000;20(4):1436-1447.

15. Rajagopalan P, Nanjappa V, Patel K, et al. Role of protein kinase N2 (PKN2) in cigarette smoke-mediated oncogenic transformation of oral cells. J Cell Commun Signal. Feb 26 2018.

16. Rohatgi N, Kaur J, Srivastava A, Ralhan R. Smokeless tobacco (khaini) extracts modulate gene expression in epithelial cell culture from an oral hyperplasia. Oral Oncol. Sep 2005;41(8):806-820.

17. Eissenberg T, Shihadeh A. Waterpipe tobacco and cigarette smoking: direct comparison of toxicant exposure. Am J Prev Med. Dec 2009;37(6):518-523.

18. Beane J, Sebastiani P, Liu G, Brody JS, Lenburg ME, Spira A. Reversible and permanent effects of tobacco smoke exposure on airway epithelial gene expression. Genome Biol. 2007;8(9):R201.

19. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. May 2012;2(5):401-404.

20. Keshava Prasad TS, Goel R, Kandasamy K, et al. Human Protein Reference Database--2009 update. Nucleic Acids Res. Jan 2009;37(Database issue):D767-772.

21. Alexandrov LB, Ju YS, Haase K, et al. Mutational signatures associated with tobacco smoking in human cancer. Science. Nov 4 2016;354(6312):618-622.

22. Mamtani R, Cheema S, Sheikh J, Al Mulla A, Lowenfels A, Maisonneuve P. Cancer risk in waterpipe smokers: a meta-analysis. Int J Public Health. Jan 2017;62(1):73-83.

23. Aoun J, Saleh N, Waked M, Salame J, Salameh P. Lung cancer correlates in Lebanese adults: a pilot case--control study. J Epidemiol Glob Health. Dec 2013;3(4):235-244.

24. Koul PA, Hajni MR, Sheikh MA, et al. Hookah smoking and lung cancer in the Kashmir valley of the Indian subcontinent. Asian Pac J Cancer Prev. 2011;12(2):519-524.

25. Al-Hebshi NN, Li S, Nasher AT, et al. Exome sequencing of oral squamous cell carcinoma in users of Arabian snuff reveals novel candidates for driver genes. Int J Cancer. Jul 15 2016;139(2):363-372.

26. Chen EG, Chen Y, Dong LL, Zhang JS. Effects of SASH1 on lung cancer cell proliferation, apoptosis, and invasion in vitro. Tumour Biol. Oct 2012;33(5):1393-1401.

27. Liu P, Morrison C, Wang L, et al. Identification of somatic mutations in non-small cell lung carcinomas using whole-exome sequencing. Carcinogenesis. Jul 2012;33(7):1270-1276.

28. Wang B, Yuan F, Kong X, Hu LD, Cai YD. Identifying Novel Candidate Genes Related to Apoptosis from a Protein-Protein Interaction Network. Comput Math Methods Med. 2015;2015:715639.

29. Shah S, King EM, Mostafa MM, Altonsy MO, Newton R. DUSP1 Maintains IRF1 and Leads to Increased Expression of IRF1-dependent Genes: A MECHANISM PROMOTING GLUCOCORTICOID INSENSITIVITY. J Biol Chem. Oct 7 2016;291(41):21802-21816.

30. Peng Q, Deng Z, Pan H, Gu L, Liu O, Tang Z. Mitogen-activated protein kinase signaling pathway in oral cancer. Oncol Lett. Feb 2018;15(2):1379-1388.

9. Figure legend

Figure 1: Transitions and transversion events identified using whole exome sequence analysis of OKF6/TERT1-Shisha cells.

Figure 2: Upstream network analysis of differentially expressed genes using Ingenuity Pathway Analysis (A) Inhibition of interferon signaling pathway **(B)** Activation of MAPK1

FIGURE 1

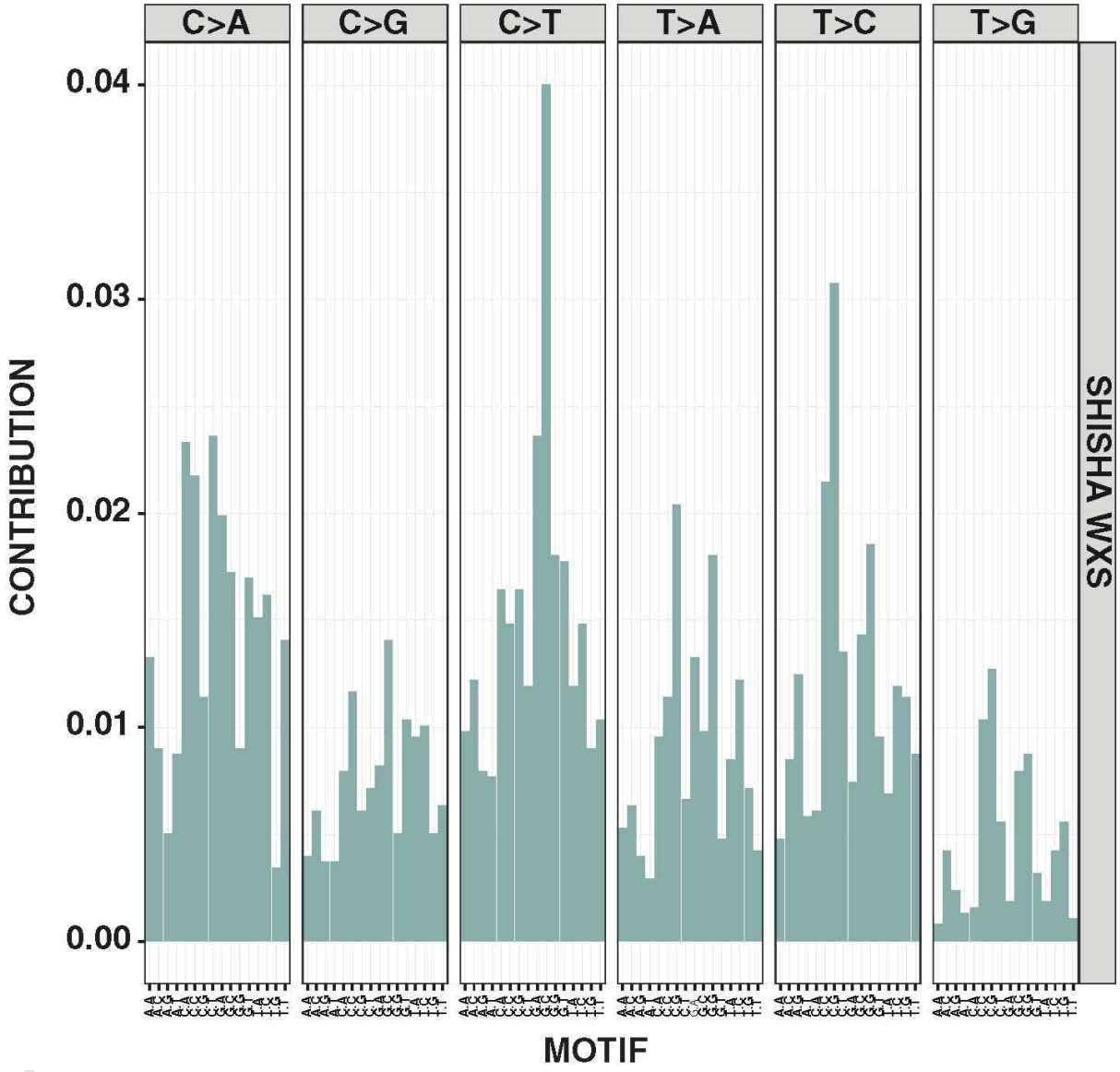

FIGURE 2