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Secretome analysis of oral keratinocytes chronically exposed to shisha

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ABSTRACT

BACKGROUND: Shisha smoking has been associated with multiple diseases including oral cancer. However, a mechanistic study to investigate alteration of secreted proteins in oral cells due to shisha smoking is lacking.

OBJECTIVES: Elucidation of differentially secreted proteins by immortalized human normal oral keratinocytes (OKF6/TERT1) upon chronic exposure to shisha.

METHODS: OKF6/TERT1 was chronically treated with 0.5% shisha extract for 8 months. Conditioned media from shisha treated (OKF6/TERT1-Shisha) and untreated (OKF6/TERT1-Parental) cells were subjected to TMT-based quantitative proteomic analysis. Bioinformatics analysis of differentially secreted proteins was carried out using SignalP, SecretomeP and TMHMM. Immunoblot validation of selected proteins was carried out to confirm the proteomics results.

RESULTS: Proteomic analysis of OKF6/TERT1–Parental and OKF6/TERT1-Shisha secretome resulted in the identification of 1,598 proteins, of which 218 proteins were found to be differentially secreted ($\geq$ 1.5-fold; p-value $\leq$ 0.05) in shisha treated cells. Bioinformatics analysis using prediction tools showed secretory potential of differentially secreted proteins identified in OKF6/TERT1-Shisha. Western blotting validated the expression of AKR1C2, HSPH1 and MMP9 in OKF6/TERT1-Shisha secretome in agreement with proteomic data.

CONCLUSION: This study serves as a useful resource to understand the effect of chronic shisha smoking on the milieu of secreted proteins of oral cells. In vivo studies are warranted to supplement our in vitro data to elucidate the role of these proteins as early diagnostic biomarkers for oral carcinogenesis among shisha smokers.

KEYWORDS: OKF6/TERT1, Secretory biomarkers, Waterpipe, TMT, Oral cancer, Proteomics

1. INTRODUCTION

Tobacco smoking has been established as a major risk factor for oral cancer. In addition to cigarette smoking, tobacco usage in the form of shisha (waterpipe) smoking has gained popularity globally [1]. The use of shisha is prevalent in the Middle Eastern countries, with a gradual shift towards younger demographic due to increasing popularity and lack of stringent regulations [2, 3]. A multi-country survey of Arab nations recorded higher preference towards shisha smoking compared to cigarette smoking or smokeless tobacco among youth [4]. Studies have demonstrated that levels of polycyclic aromatic hydrocarbons and volatile aldehydes present in mainstream shisha smoke are similar or higher compared to cigarette smoke [5, 6]. Epidemiological studies have attributed shisha smoking to lung, esophageal and gastric cancers [7-9]. However, molecular alterations associated with shisha smoking has not been well characterized [10, 11].

Secretory proteins play an important role in cell-to-cell adhesion as well as cell-matrix interactions. Analysis of secreted proteins provides insights into the nature of autocrine and paracrine signaling within the microenvironment of tissues [12]. Changes in the abundance of secretory proteins in cancers have been previously reported [13] and studying them aids in the understanding of disease progression [13, 14]. Secreted proteins are known to be involved in metastasis, and this makes secretome from tumor cells a rich reservoir for identifying potential biomarkers [15-17]. Although proteins secreted by tumors ultimately reach body fluids such as saliva and blood, their presence can be masked by more abundant proteins, making it difficult to identify and study these proteins [18, 19]. Hence, cell line-based models, which are relatively low interference systems, are preferred to understand the cancer secretome in vitro. Secretome characterization using mass spectrometry has been used for potential biomarker discovery in various cancers such as breast,

esophageal and ovarian cancers [20-22]. Candidates identified through such cell line-based discovery studies can be validated in body fluids, particularly in serum/plasma derived from cancer patients using non-invasive or minimally invasive techniques [23].

In this study, we investigated the effect of chronic shisha exposure on the secretome of normal human oral keratinocytes. We have employed mass spectrometry-based quantitative proteomics approach to identify differentially secreted proteins in normal oral keratinocytes chronically treated with shisha extract (OKF6/TERT1-Shisha) compared to untreated oral keratinocytes (OKF6/TERT1-Parental). To the best of our knowledge, this is the first study of its kind investigating the molecular alterations of secreted proteins in oral keratinocytes due to chronic shisha exposure. This study can serve as a useful resource for identification of clinically relevant early diagnostic markers for oral cancer among shisha smokers.

2. MATERIALS AND METHODS

2.1. Preparation of shisha extract and cell culture

Shisha extract was prepared using a similar method described previously by Rohatgi et al. [24]. Briefly, 5 g 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. Subsequently, the mixture was centrifuged at 12,000 rpm for 20 min and the supernatant was passed through filter papers to remove debris. The extract was sterilized using a 0.22 μm filter and stored at -80ºC until further use.

Immortalized non-transformed normal human oral keratinocyte (OKF6/TERT1) cell line was cultured in keratinocyte serum-free media (KSFM) (Life Technologies, Grand Island, NY) supplemented with bovine pituitary extract (25 mg/ml), calcium chloride (0.4 mM), epidermal growth factor (0.2 ng/ml) and 1% penicillin/streptomycin, and maintained in a humidified CO2 chamber at 37ºC.

To study the effects of shisha on oral keratinocytes, 0.5% shisha extract was added to the growth media. Fresh media along with the shisha extract was replenished every 48 hours. Cells were maintained in the culture flask until they were confluent and then passaged into fresh culture flask, and shisha treatment was repeated. The process was continued for a period of 8 months. At the end of the treatment period, cells were allowed to attain 80% confluence under shisha exposure, following which the media was removed and further steps for secretome analysis was carried out.

Cells which were treated with shisha extract were termed as 'OKF6/TERT1-Shisha'. Simultaneously, control cells which were not treated with shisha were maintained alongside for the same duration (termed as OKF6/TERT1-Parental).

2.2. Collection and processing of secretome

Secretome collection and processing was carried out as described previously by our group [25]. OKF6/TERT1-Parental and OKF6/TERT1-Shisha cells were grown to 80% confluence and washed thrice with PBS after removing the supplement-rich media. Cells were then grown in supplement-free media for 8 h. Subsequently, conditioned media was collected and centrifuged at 800 g for 10 min to remove any cell debris. The supernatant was filtered using a 0.22 μm filter and concentrated using a 3 kDa filter (Millipore, Burlington, MA).

2.3. Protein extraction, TMT labeling and bRPLC fractionation

Protein concentrations from OKF6/TERT1-Parental and OKF6/TERT1-Shisha secretome were determined using bicinchoninic acid (BCA) assay (Thermo Scientific, Bremen, Germany) [26] and equal amount of protein was collected from both conditions for further processing. Samples were reduced using dithiothreitol (DTT) at 60ºC for 20 min and alkylated using iodoacetamide (IAA) for 10 min at room temperature. The samples were then precipitated using 6 volumes of chilled acetone at -80^{o}C overnight. Following this, the samples were centrifuged for 15 min at 12,000 rpm, acetone was removed and the pellet was air-dried.

The pellets from both the treated and untreated conditions were then reconstituted in 4M urea. Protein samples were then digested using Lysyl Endopeptidase®, Mass Spectrometry Grade (Catalog#125-05061, Wako, Richmond, VA) in an enzyme-to-protein ratio of 1:100 for 4 h at 37ºC. Following this, the urea concentration was reduced from 4M to 2M using 50mM TEABC. The samples were then digested using TPCK-treated trypsin (Worthington, NJ) at an enzyme-to-protein ratio of 1:20 for 16 h at 37ºC. This was followed by sample clean-up using Sep-Pak Classic C18 columns (Catalog#WAT051910, Waters, Milford, MA). The samples were then vacuum-dried and reconstituted in 50mM TEABC for TMT-labeling.

Peptide samples were labelled using Tandem Mass Tag (TMT) labels (ThermoScientific, Bremen, Germany) as per manufacturer's instructions. Peptides derived from OKF6/TERT1-Parental and OKF6/TERT1-Shisha secretome were labelled with TMT tags 130C and 131, respectively. TMT labelled peptides were pooled and subjected to basic pH reverse phase chromatography (bRPLC), as previously described [27]. The 96 fractions obtained were concatenated into 6 fractions. The fractions were vacuum-dried, desalted using C_{18} StageTips and subjected to mass spectrometry analysis.

2.4. LC-MS^3 analysis

Peptide fractions were analysed on Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, Bremen, Germany) interfaced with Easy nLC-1000 system (Thermo Scientific, Odense, Denmark). The peptide fractions were reconstituted in 0.1% formic acid (Solvent A) and loaded onto a trap column (75 μm x 2 cm, Magic C18AQ, 5 μm, 100 Å, Michrom Biosciences Inc.,

Auburn, CA) at a flow rate of 3 µl/min. The peptides were then resolved on an in-house packed analytical column 75 µm x 20 cm, Magic C18 AQ, 3 µm, 100 Å, Michrom Biosciences Inc., Auburn, CA) at a flow rate of 300 nl/min with back pressure of 275 bar using a gradient of 10% – 35% solvent B (0.1% formic acid in 95% acetonitrile) for 100 min.

Data acquisition on the Orbitrap Fusion was carried out using a data-dependent method with synchronous precursor selection MS3 scanning for TMT tags. The scan sequence was started with the acquisition of a full MS or MS1 one spectrum acquired in the Orbitrap (m/z range, 500-1600; resolution, $1.2x10^5$; AGC target, $4x10^5$; maximum injection time, 50 ms). MS2 scans were done in the linear ion trap using the following settings: quadrupole isolation at an isolation width of 2 m/z; fragmentation method, CID; normalized collision energy, 35%. Using synchronous precursor selection the 10 most abundant fragment ions were selected for the MS3 experiment following each MS2 scan. The fragment ions were further fragmented using the HCD fragmentation (normalized collision energy, 55%) and the MS3 spectrum was acquired in the Orbitrap (m/z range, 110-550; resolution, $5x10^4$; AGC target, $5x10^4$; maximum injection time, 100 ms). The peptide fractions were analysed in triplicate on the mass spectrometer.

2.5. Data analysis

Identification and quantification of proteins was carried out using Proteome Discoverer (version 2.1) software suite (Thermo Scientific, Bremen, Germany). Raw files were searched against NCBI human RefSeq protein database (version 75) containing common contaminants, using Sequest HT search algorithm. In the search parameters, carbamidomethylation of cysteine, and TMT label at peptide N-termini and lysine were set as static modifications; while oxidation of methionine was

set as a dynamic modification. Trypsin was chosen as the protease with a maximum allowed missed cleavage of 1. Precursor mass tolerance was set to 10 ppm and fragment mass tolerance was set to 0.1 Da. Decoy database search was carried out and peptide spectrum matches with a false discovery rate (FDR) cut-off of 1% was considered for peptide identification. Quantitative node in Proteome Discoverer was used to calculate protein ratios between OKF6/TERT1-Shisha and OKF6/TERT1-Parental using reporter ion intensities. Protein fold-change ratio was calculated as 131/130C (OKF6/TERT1-Shisha / OKF6/TERT1-Parental). Scatter plots were generated using $\log_2$ fold change ratio between replicates. Pearson correlation coefficient (r) was then calculated to evaluate the linear relationship between the technical replicates. A volcano plot was generated to depict the distribution of proteins based on their fold change and corresponding p-value.

2.6. Bioinformatics analysis

Proteins identified in conditioned media were assessed for their secretory potential using SignalP [28]. Proteins following non-classical secretion pathways were predicted using SecretomeP, and TMHMM was used to predict transmembrane domain [29, 30]. Proteome of conditioned media was also compared with experimentally annotated databases - ExoCarta [31], Vesiclepedia [32] and Human Protein Atlas (HPA) [33] to identify vesicular proteins based on experimental evidence. In addition, the data was compared with the updated salivary proteome [34] to identify differentially secreted proteins potentially detectable in saliva.

2.7 Immunoblot assay

30 µg equivalent protein lysate was taken from OKF6/TERT1-Parental and OKF6/TERT1-Shisha secretome, and western blots were carried out as described earlier [25]. Proteins were resolved using polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on to nitrocellulose membrane at 200 mA for 2 h. Membrane was blocked with 5% non-fat dry milk solution prepared in phosphate buffered saline containing 0.1% Tween-20 at room temperature for 30 min. The membrane was then probed with rabbit polyclonal anti-AKR1C2 (Catalog#PA001543HA01HU; Cusabio, Houston, TX), rabbit polyclonal anti-HSPH1 (Catalog# HPA028675; Sigma Aldrich, St. Louis, MO) and rabbit polyclonal anti-MMP9 (Catalog#137867; Abcam). TIMP1, whose expression in OKF6/TERT1-Shisha secretome was unchanged compared to parental secretome, was used as loading control and probed with rabbit polyclonal anti-TIMP1 antibody (Catalog# PA023560YA01HU, Cusabio).

2.8. Statistical analysis

Statistical significance was calculated in Perseus software suit [35] using student's t-test.

2.9. Data availability

Proteomics data has been submitted to ProteomeXchange Consortium (http://www.proteomecentral.proteomexchange.org) via the PRIDE public data repository [36] and can be accessed using the data identifier- PXD011130.

3. RESULTS

3.1. Quantitative proteomic analysis of OKF6/TERT1-Shisha secretome

Mass spectrometry-based quantitative proteomic analysis of secretome from OKF6/TERT1-Parental and OKF6/TERT1-Shisha cells resulted in the identification of 1,598 proteins. The workflow employed in this study is depicted in **Fig 1A**. High correlation ($r \geq 0.96$) of protein fold changes (OKF6/TERT1-Shisha / OKF6/TERT1-Parental) were observed across replicates in the proteomic data (**Fig 1B**).Volcano plot showing protein fold change distribution of all identified proteins and their corresponding p-values is shown in **Fig 1C**. Of the total identified proteins, we observed 218 proteins to be differentially secreted ($\geq$ 1.5-fold; p-value ≤ 0.05) in OKF6/TERT1-Shisha secretome compared to parental secretome across all three replicates. Among these, 112 proteins were found to be secreted in higher abundance (fold change ≥ 1.5) and 106 proteins were found to be secreted in lower abundance (fold change ≤ 0.66 fold) in OKF6/TERT1-Shisha secretome compared to parental secretome. A partial list of differentially secreted proteins identified in this study is provided in **Table 1. Fig 2** depicts representative MS/MS spectra for selected differentially secreted proteins, aldo-keto reductase family 1 member C2 (AKR1C2), galectin-1 (LGALS1), heat shock protein family H (Hsp110) member 1 (HSPH1) and matrix metallopeptidase (MMP9), which are frequently reported to be differentially expressed in cancers [37-41]. Complete list of proteins and peptides identified in this study are provided in **Supplementary Table 1**.

3.2. Bioinformatics analysis of dysregulated proteins in OKF6/TERT1-Shisha secretome

Differentially secreted proteins identified in our data were analyzed for secretory potential using prediction-based bioinformatics tools (SignalP and SecretomeP) and by confirmation with databases of experimentally validated secreted proteins (Vesiclepedia, ExoCarta and HPA). Overall, we found secretory evidence for 214 out of 218 differentially secreted proteins in OKF6/TERT1-Shisha (**Fig 3A**). Comparison of differentially secreted proteins with the secreted protein-database in Human Protein Atlas showed an overlap of 57 proteins. In addition, we also compared the differentially secreted proteins in our data with ExoCarta and Vesiclepedia databases to identify exosomal proteins which are released into extracellular space in the form of membranous vesicles. In total, we found 208 proteins with experimental evidence for vesicle-mediated exosomal secretion (**Fig 3B, Supplementary Table 2**).

We further analyzed the differentially secreted proteins for the presence of N-terminal signal peptides using SignalP software. Proteins which contain N-terminal hydrophobic peptides are released from cells through the classical secretory pathway facilitated by endoplasmic reticulum [42]. Our analysis predicted 61 differentially secreted proteins contain N-terminal signal peptide. In addition, based on SecretomeP analysis, we identified 43 proteins differentially secreted in OKF6/TERT1-Shisha secretome which do not contain a signal peptide but are predicted to be secreted by non-classical pathways (**Fig 3C, Supplementary Table 2**). Further, we analyzed proteins for the presence of a transmembrane domain using TMHMM analysis. We identified 25 differentially secreted proteins in OKF6/TERT1-Shisha secretome predicted to contain a transmembrane helical domain (**Supplementary Table 2**).

We evaluated differentially secreted proteins in shisha exposed cells (compared to parental cells) that have been previously detected in normal human saliva. We compared differentially secreted proteins with the salivary proteome study which cataloged the normal human salivary proteins based on mass spectrometry evidence [34]. We found that 167 proteins differentially secreted in our data overlapped with proteins identified in human saliva, of which 89 proteins were secreted in higher abundance in OKF6/TERT1-Shisha secretome (**Fig 3D, Supplementary Table 2**). Taken together, our analyses provides strong evidence of secretory potential for the differentially secreted proteins in our data. In addition, comparison of higher abundant proteins identified in our data with the salivary proteome serves the objective of this study to identify early diagnostic biomarkers for oral cancer in shisha smokers.

3.3. Identification of potential cancer biomarkers using the OKF6/TERT1-Shisha secretome data

Based on proteomic evidence, we established that normal oral keratinocytes OKF6/TERT1 chronically treated with shisha extract results in altered secretion of proteins. We compared the differentially secreted proteins identified in our data with four HNSCC studies published previously. Marimuthu et. al. carried out iTRAQ-based quantitative proteomic analysis of the secretome of 6 HNSCC cell lines – JHU-O11, JHU-O22, JHU-O28, JHU-O29, FaDu and CAL 27, and compared with the secretome of OKF6/TERT1 [25]. Sepiashvili et. al. studied the secretome of HNSCC cell lines FaDu, UTSCC8 and UTSCC42a using mass spectrometry-based proteomic approach and gene expression microarrays [43]. Yu et. al. carried out secretome analysis of OSCC cell lines OECM1 and SCC4, using mass spectrometry-based approach and further validated their

findings with tissue transcriptome analysis [44]. Rahlan et. al. employed proteomics approach to identify proteins in the secretome of four HNSCC cell lines- SCC4, HSC2, SCC38, and AMOSIII [45]. Upon comparison, we found that 134, 131, 21 and 18 proteins reported to be differentially secreted (≥ 1.5-fold) by Marimuthu et. al., Yu et. al., Rahlan et. al. and Sepiashvili et. al. respectively, overlapped with the differentially secreted proteins identified in OKF6/TERT1-Shisha secretome (**Fig 4A, Supplementary Table 2).** These include higher abundance proteins such as transforming protein RhoA (RHOA) and eukaryotic elongation factors (EEF1G, EEF2. EEF1G) and lower abundance proteins in our data such as endoplasmic reticulum resident protein 29 (ERP29) and secreted protein acidic and rich in cysteine (SPARC), whose expression in OKF6/TERT1-Shisha secretome correlated with the aforementioned HNSCC secretome studies.

Differentially secreted proteins in our data and those reported previously in cancer cell lines indicate that chronic exposure of normal oral keratinocytes to shisha may lead to cellular transformation. Identification of such proteins will facilitate identification of early oral cancer diagnostic biomarkers among shisha users.

3.4 Immunoblot validation of differentially secreted proteins identified in OKF6/TERT1-Shisha secretome

To confirm the results of proteomic analysis, we carried out western blot validation of differentially secreted proteins identified in OKF6/TERT1-Shisha. These proteins were also reported to be differentially secreted in studies pertaining to HNSCC secretome as discussed above. In agreement with our mass spectrometry data, we observed significantly increased

abundance of AKR1C2 and HSPH1 and decreased abundance of MMP9 compared to OKF6/TERT1-Parental secretome (**Fig 4B)**.

DISCUSSION

Shisha smoking has been a recreational practice in the Middle Eastern and South East Asian countries since many centuries. However, emergence of shisha smoking among Western nations has been observed recently [46, 47]. Despite being categorized as a health hazard, the pathobiology of shisha smoking-related diseases remains poorly understood. Analysis of secreted proteins in disease conditions has the potential to reveal biomarkers that could be monitored in body fluids. Particularly in the case of oral cancer and head and neck cancers, where tobacco smoking remains a predominant risk factor, identification of potential prognostic biomarkers in saliva and serum have proven to be clinically relevant [48, 49]. In this study, we investigated the effects of chronic shisha exposure on secreted proteins in oral keratinocytes. We chronically treated normal immortalized non-transformed human oral keratinocytes (OKF6/TERT1) with shisha extract for a period of 8 months (OKF6/TERT1-Shisha), followed by proteomic analysis of the secretome which was compared with that of untreated cells (OKF6/TERT1-Parental).

Mass spectrometry-based quantitative proteomic analysis of OKF6/TERT1-Shisha secretome resulted in the identification of 218 differentially secreted proteins ($p \leq 0.05$). We identified several proteins which have been previously identified in oral cancer. For example, AKR1C2 is an enzyme involved in sterol metabolism and catalyzes the conversion of aldehydes and ketones to their corresponding alcohols. A study by Wenners et al., have examined the expression of AKR1C1 and AKR1C2 in breast cancer tissue and have established elevated expression in the stromal region of tumor samples [50]. Microarray-based gene expression study by Woo et. al. detected higher expression of AKR1C2 in OSCC cell lines exposed to cigarette smoke and particulate matter [51].

We have also identified decreased abundance of Galectin-1 in our data. A study recently on colorectal cancer reported the silencing of galectin-1 expression due to promoter hypermethylation which contributes to cellular proliferation and escape apoptosis [41]. Our study also reports the higher abundance of HSPH1 in the secretome of shisha-treated OKF6/TERT1 cells. HSPH1 is a molecular chaperone which prevents the aggregation of denatured proteins and misfolding of other heat shock proteins. HSPH1 has been reported as one of the many heat shock proteins detected to be abundant in the conditioned media of colon cancer cell lines [52]. A recent study carried out by our own group has reported the overexpression of HSPH1 in HNSCC cell lines CAL 27, FaDu and JHU-O28 in comparison with OKF6/TERT1 [53]. Furthermore, among the differentially secreted proteins we observed lower abundance of MMP9 in OKF6/TERT1-Shisha secretome compared to parental secretome. While certain studies have indicated the oncogenic properties of metallopeptidases, animal model studies based on MMP-9 knockdown have also observed increased aggressiveness in tumors [54]. A recent study reported that serum levels of MMP2 and MMP9 were associated with favorable outcomes in breast cancer patients and had better response to chemotherapy [55]. Watson et. al. investigated the expression of MMP9 and TIMP1 among the secreted proteins in human respiratory epithelia exposed to cigarette smoke and reported a reduced abundance of MMP9 due to smoke exposure [56]. Similar observations are reported on GAPDH, which was observed to be highly abundant in OKF6/TERT1-Shisha secretome. GAPDH is known to play a central role in the glycolysis pathway but its aberrant expression has also been implicated to regulate growth, proliferation and apoptosis in cancer cells [57]. A population-based study has identified overexpression of GAPDH in the salivary samples of patients diagnosed with oral squamous cell carcinoma [58]. Similarly, increased levels of GAPDH have been detected in preoperative blood samples of laryngeal carcinoma patients [59]. Such studies show correlation

between differentially secreted proteins identified in our shisha-exposed model and various disease conditions indicating the detrimental effects of chronic exposure to shisha.

We compared out data with previous studies pertaining to HNSCC secretome and observed an overlap of several proteins differentially secreted by OKF6/TERT1-Shisha cells. We observed an increased abundance of pyruvate kinase M1/2 (PKM) in our data, which is known to facilitate adhesion and migration of colon cancer cells by regulating STAT-3 associated signaling [60]. Similarly, our data also shows increased abundance of two eukaryotic elongation factors, EEF1G and EEF2. EEF1G is known to interact with E-cadherin to promote cell adhesion and has been demonstrated to be highly abundant both at mRNA and protein levels in colorectal carcinoma [61, 62]. Secretome of OKF6/TERT1-Shisha cells was found to have higher abundance of proteins reported in cell proliferation including transforming protein RhoA (RHOA) and prostaglandin E synthase 3 (PTGES3) amongst others. Overexpression of RHOA has been reported to promote proliferation and migration of cervical cancer cells [63]. Similarly, studies have shown increased expression of PTGES3 in the oral mucosa of smokers and hypoxia-induced esophageal squamous cell carcinoma (ESCC) [64, 65]. Comparison with published secretome studies revealed that PKM1/2, EEF1G, EEF2, RHOA and PTGES3 are differentially secreted in HNSCC cells and have also been identified in human saliva [34, 35].

Similarly, we detected higher abundance of chaperone proteins such as endoplasmin (HSP90B1), heat shock protein family H (Hsp110) member 1 (HSPH1) which have been reported in several cancers previously [66]. In addition, we also detected decreased abundance of ECM proteins such as matrix metallopeptidase 9 (MMP9), secreted protein acidic and rich in cysteine (SPARC) and

cysteine rich angiogenic inducer 61 (CYR61) among others in OKF6/TERT1-Shisha secretome. Dysregulation of certain ECM proteins has been known to promote detachment of cancer cells from their primary tumor and to facilitate metastasis [67, 68]. Expression of SPARC and CYR61 inversely correlates with cell invasion and metastasis in ovarian, prostate and gastric cancer, respectively [69-72]. These findings indicate that chronic shisha exposure alters the expression of secreted proteins involved in cellular processes linked to tumorigenesis.

Our data also suggests alteration in metabolic pathways in OKF6/TERT1 cells chronically treated with shisha extract. Aberrant metabolism has been widely established as one of the hallmarks of cancer and is often associated with increased energy requirements and requirement of metabolites for anabolic pathways [73]. Although conventionally believed to be restricted to the cytoplasm, studies have demonstrated that glycolytic enzymes have non-canonical extracellular functions as well in cancers, such as cytokine signaling and anti-apoptotic response [74]. Moreover, elevated levels of metabolic enzymes in the serum have been correlated with tumorigenesis and drug resistance [75, 76]. Increased secretion of glycolytic proteins GAPDH, PFKP and GPI has been previously reported in HNSCC secretome [12, 25, 29, 31].

In summary, this study highlights the detrimental effects of shisha smoking on oral keratinocytes in vitro and provides list of candidate proteins that potentially play a role in oral carcinogenesis. We hypothesize that differentially secreted proteins identified in this study may serve as early diagnostic markers among shisha smokers. Further studies using salivary samples from OSCC patients with shisha smoking habits are warranted to establish these proteins as clinically relevant biomarkers.

CONCLUSIONS

The current study was carried out to investigate the adverse effects of chronic exposure of shisha in oral keratinocytes by studying their secretory protein profile. Using proteomics-based approach, we identified several differentially secreted proteins in the secretome of shisha-exposed cells. Our data indicates that chronic exposure to shisha results in aberrant oxidative stress response and altered glycolytic pathway in oral keratinocytes. Comparison of our data with previously published OSCC and HNSCC secretome studies showed an overlap of several differentially secreted proteins, many of which were also detectable in human saliva. This study demonstrates molecular aberrations in oral keratinocytes upon chronic shisha exposure and highlights the potential of several identified secretory proteins as biomarkers in oncogenic risk assessment amongst shisha smokers.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

REFERENCES

[1] Maziak W, Taleb ZB, Bahelah R, et al. The global epidemiology of waterpipe smoking. Tob Control. 2015;24 Suppl 1:i3-i12.

[2] Ramji R, Arnetz BB, Nilsson M, et al. Waterpipe use in adolescents in Northern Sweden: Association with mental well-being and risk and health behaviours. Scand J Public Health. 2017:1403494817746534.

[3] Maziak W. The waterpipe: a new way of hooking youth on tobacco. Am J Addict. 2014;23(2):103-7.

[4] Moh'd Al-Mulla A, Abdou Helmy S, Al-Lawati J, et al. Prevalence of tobacco use among students aged 13-15 years in Health Ministers' Council/Gulf Cooperation Council Member States, 2001-2004. J Sch Health. 2008;78(6):337-43.

[5] Sepetdjian E, Shihadeh A, Saliba NA. Measurement of 16 polycyclic aromatic hydrocarbons in narghile waterpipe tobacco smoke. Food Chem Toxicol. 2008;46(5):1582-90.

[6] Al Rashidi M, Shihadeh A, Saliba NA. Volatile aldehydes in the mainstream smoke of the narghile waterpipe. Food Chem Toxicol. 2008;46(11):3546-9.

[7] Dar NA, Bhat GA, Shah IA, et al. Hookah smoking, nass chewing, and oesophageal squamous cell carcinoma in Kashmir, India. Br J Cancer. 2012;107(9):1618-23.

[8] Sadjadi A, Derakhshan MH, Yazdanbod A, et al. Neglected role of hookah and opium in gastric carcinogenesis: a cohort study on risk factors and attributable fractions. Int J Cancer. 2014;134(1):181-8.

[9] Gupta D, Boffetta P, Gaborieau V, et al. Risk factors of lung cancer in Chandigarh, India. Indian J Med Res. 2001;113:142-50.

[10] El-Hakim IE, Uthman MA. Squamous cell carcinoma and keratoacanthoma of the lower lip associated with "Goza" and "Shisha" smoking. Int J Dermatol. 1999;38(2):108-10.

[11] Cobb C, Ward KD, Maziak W, et al. Waterpipe tobacco smoking: an emerging health crisis in the United States. Am J Health Behav. 2010;34(3):275-85.

[12] Barlowe CK, Miller EA. Secretory protein biogenesis and traffic in the early secretory pathway. Genetics. 2013;193(2):383-410.

[13] Kunz-Schughart LA, Knuechel R. Tumor-associated fibroblasts (part I): Active stromal participants in tumor development and progression? Histol Histopathol. 2002;17(2):599-621.

[14] Liao Z, Tan ZW, Zhu P, et al. Cancer-associated fibroblasts in tumor microenvironment - Accomplices in tumor malignancy. Cell Immunol. 2018.

[15] Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001;410(6824):50-6.

[16] Nomura T, Katunuma N. Involvement of cathepsins in the invasion, metastasis and proliferation of cancer cells. J Med Invest. 2005;52(1-2):1-9.

[17] Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2002;2(3):161-74.

[18] Xue H, Lu B, Lai M. The cancer secretome: a reservoir of biomarkers. J Transl Med. 2008;6:52.

[19] Tonack S, Aspinall-O'Dea M, Jenkins RE, et al. A technically detailed and pragmatic protocol for quantitative serum proteomics using iTRAQ. J Proteomics. 2009;73(2):352-6.

[20] Gunawardana CG, Kuk C, Smith CR, et al. Comprehensive analysis of conditioned media from ovarian cancer cell lines identifies novel candidate markers of epithelial ovarian cancer. J Proteome Res. 2009;8(10):4705-13.

[21] Kulasingam V, Diamandis EP. Proteomics analysis of conditioned media from three breast cancer cell lines: a mine for biomarkers and therapeutic targets. Mol Cell Proteomics. 2007;6(11):1997-2011.

[22] Kashyap MK, Harsha HC, Renuse S, et al. SILAC-based quantitative proteomic approach to identify potential biomarkers from the esophageal squamous cell carcinoma secretome. Cancer Biol Ther. 2010;10(8):796-810.

[23] Makridakis M, Vlahou A. Secretome proteomics for discovery of cancer biomarkers. J Proteomics. 2010;73(12):2291-305.

[24] Rohatgi N, Kaur J, Srivastava A, et al. Smokeless tobacco (khaini) extracts modulate gene expression in epithelial cell culture from an oral hyperplasia. Oral Oncol. 2005;41(8):806-20.

[25] Marimuthu A, Chavan S, Sathe G, et al. Identification of head and neck squamous cell carcinoma biomarker candidates through proteomic analysis of cancer cell secretome. Biochim Biophys Acta. 2013;1834(11):2308-16.

[26] Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985;150(1):76-85.

[27] Radhakrishnan A, Nanjappa V, Raja R, et al. A dual specificity kinase, DYRK1A, as a potential therapeutic target for head and neck squamous cell carcinoma. Sci Rep. 2016;6:36132.

[28] Petersen TN, Brunak S, von Heijne G, et al. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011;8(10):785-6.

[29] Krogh A, Larsson B, von Heijne G, et al. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 2001;305(3):567-80.

[30] Bendtsen JD, Jensen LJ, Blom N, et al. Feature-based prediction of non-classical and leaderless protein secretion. Protein Eng Des Sel. 2004;17(4):349-56.

[31] Keerthikumar S, Chisanga D, Ariyaratne D, et al. ExoCarta: A Web-Based Compendium of Exosomal Cargo. J Mol Biol. 2016;428(4):688-92.

[32] Kalra H, Simpson RJ, Ji H, et al. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. PLoS Biol. 2012;10(12):e1001450.

[33] Thul PJ, Akesson L, Wiking M, et al. A subcellular map of the human proteome. Science. 2017;356(6340).

[34] Sivadasan P, Gupta MK, Sathe GJ, et al. Human salivary proteome--a resource of potential biomarkers for oral cancer. J Proteomics. 2015;127(Pt A):89-95.

[35] Tyanova S, Temu T, Sinitcyn P, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods. 2016;13(9):731-40.

[36] Vizcaino JA, Deutsch EW, Wang R, et al. ProteomeXchange provides globally coordinated proteomics data submission and dissemination. Nat Biotechnol. 2014;32(3):223-6.

[37] Li W, Hou G, Zhou D, et al. The roles of AKR1C1 and AKR1C2 in ethyl-3,4-dihydroxybenzoate induced esophageal squamous cell carcinoma cell death. Oncotarget. 2016;7(16):21542-55.

[38] Arif M, Vedamurthy BM, Choudhari R, et al. Nitric oxide-mediated histone hyperacetylation in oral cancer: target for a water-soluble HAT inhibitor, CTK7A. Chem Biol. 2010;17(8):903-13.

[39] Kai M, Nakatsura T, Egami H, et al. Heat shock protein 105 is overexpressed in a variety of human tumors. Oncol Rep. 2003;10(6):1777-82.

[40] Vilen ST, Salo T, Sorsa T, et al. Fluctuating roles of matrix metalloproteinase-9 in oral squamous cell carcinoma. ScientificWorldJournal. 2013;2013:920595.

[41] Satelli A, Rao US. Galectin-1 is silenced by promoter hypermethylation and its re-expression induces apoptosis in human colorectal cancer cells. Cancer Lett. 2011;301(1):38-46.

[42] von Heijne G. Signal sequences. The limits of variation. J Mol Biol. 1985;184(1):99-105.

[43] Sepiashvili L, Hui A, Ignatchenko V, et al. Potentially novel candidate biomarkers for head and neck squamous cell carcinoma identified using an integrated cell line-based discovery strategy. Mol Cell Proteomics. 2012;11(11):1404-15.

[44] Yu CJ, Chang KP, Chang YJ, et al. Identification of guanylate-binding protein 1 as a potential oral cancer marker involved in cell invasion using omics-based analysis. J Proteome Res. 2011;10(8):3778-88.

[45] Ralhan R, Masui O, Desouza LV, et al. Identification of proteins secreted by head and neck cancer cell lines using LC-MS/MS: Strategy for discovery of candidate serological biomarkers. Proteomics. 2011;11(12):2363-76.

[46] Maziak W, Nakkash R, Bahelah R, et al. Tobacco in the Arab world: old and new epidemics amidst policy paralysis. Health Policy Plan. 2014;29(6):784-94.

[47] Leung JM, Sin DD. Smoke and mirrors: the perils of water-pipe smoking and implications for Western countries. Chest. 2014;146(4):875-6.

[48] Nosratzehi T. Salivary Chemical Factors in Relation with Oral Cancer in Smokers and Non-Smokers: a Literature Review. J Dent (Shiraz). 2017;18(4):237-43.

[49] Franzmann EJ, Reategui EP, Pereira LH, et al. Salivary protein and solCD44 levels as a potential screening tool for early detection of head and neck squamous cell carcinoma. Head Neck. 2012;34(5):687-95.

[50] Wenners A, Hartmann F, Jochens A, et al. Stromal markers AKR1C1 and AKR1C2 are prognostic factors in primary human breast cancer. Int J Clin Oncol. 2016;21(3):548-56.

[51] Woo S, Gao H, Henderson D, et al. AKR1C1 as a Biomarker for Differentiating the Biological Effects of Combustible from Non-Combustible Tobacco Products. Genes (Basel). 2017;8(5).

[52] Imperlini E, Colavita I, Caterino M, et al. The secretome signature of colon cancer cell lines. J Cell Biochem. 2013;114(11):2577-87.

[53] Babu N, Mohan S, Nanjappa V, et al. Identification of potential biomarkers of head and neck squamous cell carcinoma using iTRAQ based quantitative proteomic approach. Data in Brief. 2018;19:1124-30.

[54] Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev. 2006;25(1):9-34.

[55] Tabouret E, Bertucci F, Pierga JY, et al. MMP2 and MMP9 serum levels are associated with favorable outcome in patients with inflammatory breast cancer treated with bevacizumab-based neoadjuvant chemotherapy in the BEVERLY-2 study. Oncotarget. 2016;7(14):18531-40.

[56] Watson AM, Benton AS, Rose MC, et al. Cigarette smoke alters tissue inhibitor of metalloproteinase 1 and matrix metalloproteinase 9 levels in the basolateral secretions of human asthmatic bronchial epithelium in vitro. J Investig Med. 2010;58(5):725-9.

[57] Krasnov GS, Dmitriev AA, Snezhkina AV, et al. Deregulation of glycolysis in cancer: glyceraldehyde-3-phosphate dehydrogenase as a therapeutic target. Expert Opin Ther Targets. 2013;17(6):681-93.

[58] Gleber-Netto FO, Yakob M, Li F, et al. Salivary Biomarkers for Detection of Oral Squamous Cell Carcinoma in a Taiwanese Population. Clin Cancer Res. 2016;22(13):3340-7.

[59] Garcia-Olmo DC, Contreras JD, Picazo MG, et al. Potential clinical significance of perioperative levels of mRNA in plasma from patients with cancer of the larynx or hypopharynx. Head Neck. 2017;39(4):647-55.

[60] Yang P, Li Z, Fu R, et al. Pyruvate kinase M2 facilitates colon cancer cell migration via the modulation of STAT3 signalling. Cell Signal. 2014;26(9):1853-62.

[61] Guo Z, Neilson LJ, Zhong H, et al. E-cadherin interactome complexity and robustness resolved by quantitative proteomics. Sci Signal. 2014;7(354):rs7.

[62] Mathur S, Cleary KR, Inamdar N, et al. Overexpression of elongation factor-1gamma protein in colorectal carcinoma. Cancer. 1998;82(5):816-21.

[63] Liu X, Chen D, Liu G. Overexpression of RhoA promotes the proliferation and migration of cervical cancer cells. Biosci Biotechnol Biochem. 2014;78(11):1895-901.

[64] Lee JJ, Natsuizaka M, Ohashi S, et al. Hypoxia activates the cyclooxygenase-2-prostaglandin E synthase axis. Carcinogenesis. 2010;31(3):427-34.

[65] Boyle JO, Gumus ZH, Kacker A, et al. Effects of cigarette smoke on the human oral mucosal transcriptome. Cancer Prev Res (Phila). 2010;3(3):266-78.

[66] Santos TG, Martins VR, Hajj GNM. Unconventional Secretion of Heat Shock Proteins in Cancer. Int J Mol Sci. 2017;18(5).

[67] Hasan NM, Adams GE, Joiner MC, et al. Hypoxia facilitates tumour cell detachment by reducing expression of surface adhesion molecules and adhesion to extracellular matrices without loss of cell viability. Br J Cancer. 1998;77(11):1799-805.

[68] Buchheit CL, Weigel KJ, Schafer ZT. Cancer cell survival during detachment from the ECM: multiple barriers to tumour progression. Nat Rev Cancer. 2014;14(9):632-41.

[69] de Groote ML, Kazemier HG, Huisman C, et al. Upregulation of endogenous ICAM-1 reduces ovarian cancer cell growth in the absence of immune cells. Int J Cancer. 2014;134(2):280-90.

[70] Donadio AC, Remedi MM, Frede S, et al. Decreased expression of intercellular adhesion molecule-1 (ICAM-1) and urokinase-type plasminogen activator receptor (uPAR) is associated with tumor cell spreading in vivo. Clin Exp Metastasis. 2002;19(5):437-44.

[71] D'Antonio KB, Schultz L, Albadine R, et al. Decreased expression of Cyr61 is associated with prostate cancer recurrence after surgical treatment. Clin Cancer Res. 2010;16(23):5908-13.

[72] Zhang J, Wang P, Zhu J, et al. SPARC expression is negatively correlated with clinicopathological factors of gastric cancer and inhibits malignancy of gastric cancer cells. Oncol Rep. 2014;31(5):2312-20.

[73] Liberti MV, Locasale JW. The Warburg Effect: How Does it Benefit Cancer Cells? Trends Biochem Sci. 2016;41(3):211-8.

[74] Yu X, Li S. Non-metabolic functions of glycolytic enzymes in tumorigenesis. Oncogene. 2017;36(19):2629-36.

[75] Gault MH, Cohen MW, Kahana LM, et al. Serum enzymes in patients with carcinoma of lung: lactic-acid dehydrogenase, phosphohexose isomerase, alkaline phosphatase and glutamic oxaloacetic transaminase. Can Med Assoc J. 1967;96(2):87-94.

[76] Shin YK, Yoo BC, Hong YS, et al. Upregulation of glycolytic enzymes in proteins secreted from human colon cancer cells with 5-fluorouracil resistance. Electrophoresis. 2009;30(12):2182-92.

[77] Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37(1):1-13.

[78] Okegawa T, Pong RC, Li Y, et al. The role of cell adhesion molecule in cancer progression and its application in cancer therapy. Acta Biochim Pol. 2004;51(2):445-57.

LIST OF TABLES

Table 1. Partial list of differentially secreted proteins identified in OKF6/TERT1 cells chronically treated with shisha extract

Gene symbol	Protein name	Median fold change (OKF6/TERT1-Shisha / OKF6/TERT1-Parental)	SignalP status	SecretomeP status	TMHMM status	Identified previously in HNSCC secretome
GPC1	Glypican-1	2.0	Yes	N/A	N/A	[12, 44, 77]
LDHA	L-lactate dehydrogenase A	1.9	N/A	N/A	N/A	[25, 44, 45, 77]
C3	Complement C3	1.8	Yes	N/A	N/A	[12, 44, 77]
ACTN4	Alpha actinin-4	1.7	N/A	Yes	N/A	[25, 44, 45, 77]
LGALS1	Galectin-1	0.6	N/A	N/A	N/A	[78]
PFN1	Profilin-1	0.5	N/A	N/A	N/A	[78]
FN1	Fibronectin	0.4	Yes	N/A	N/A	[77_ENREF_77, 78]

Supplementary Table 1: List of proteins identified and quantified in OKF6/TERT1-Shisha secretome and OKF6/TERT1-Parental secretome using TMT-based quantitative proteomics approach

Supplementary Table 2: List of differentially secreted proteins identified in OKF6/TERT1-Shisha secretome compared to OKF6/TERT1-Parental secretome

LIST OF FIGURES

Figure 1. Quantitative proteomic analysis of OKF6/TERT1-Shisha secretome. (**A**) Workflow for TMT-based quantitative secretome analysis of OKF6/TERT1 cells chronically treated with 0.5% shisha extract. (**B**) Scatter plots representing the correlation of protein fold change (OKF6/TERT1-Shisha / OKF6/TERT1-Parental) across three replicates. Pearson correlation coefficient (r) was calculated to evaluate the linear relationship between the technical replicates ($r \geq 0.96$). (**C**) Volcano plot showing the distribution of proteins identified in this study as a measure of their fold change (OKF6/TERT1-Shisha / OKF6/TERT1-Parental) versus p-value.

Figure 2. Representative MS/MS spectra of proteins found to be differentially abundant in OKF6/TERT1-Shisha secretome – (A) AKR1C2 (B) LGALS1 (C) HSPH1 (D) MMP9. Inset peaks represent tandem mass tag (TMT) label intensities representing fold change of the protein.

Figure 3. Secretory evidence for differentially abundant proteins in OKF6/TERT1-Shisha secretome. (A) Pie-Chart representation of the number of differentially secreted proteins identified in this study which have experimental and prediction-based evidence for secretory potential. (B) Venn diagram depicting the distribution of differentially secreted proteins identified in this study compared with experimentally validated databases. (C) Venn diagram depicting the differentially secreted proteins identified this study with secretory potential analyzed using prediction-based tools – SignalP and SecretomeP (D) Venn diagram representing the number of proteins differentially abundant in this study reported to be present in normal human salivary proteome (Sivadasan et. al., 2015).

Figure 4. (A) Venn diagram representing differentially secreted proteins identified in this study as compared to proteins reported in published HNSCC/OSCC secretome studies. (B) Western blot validation for selected proteins differentially secreted in OKF6/TERT1-Shisha secretome.

Figure 1

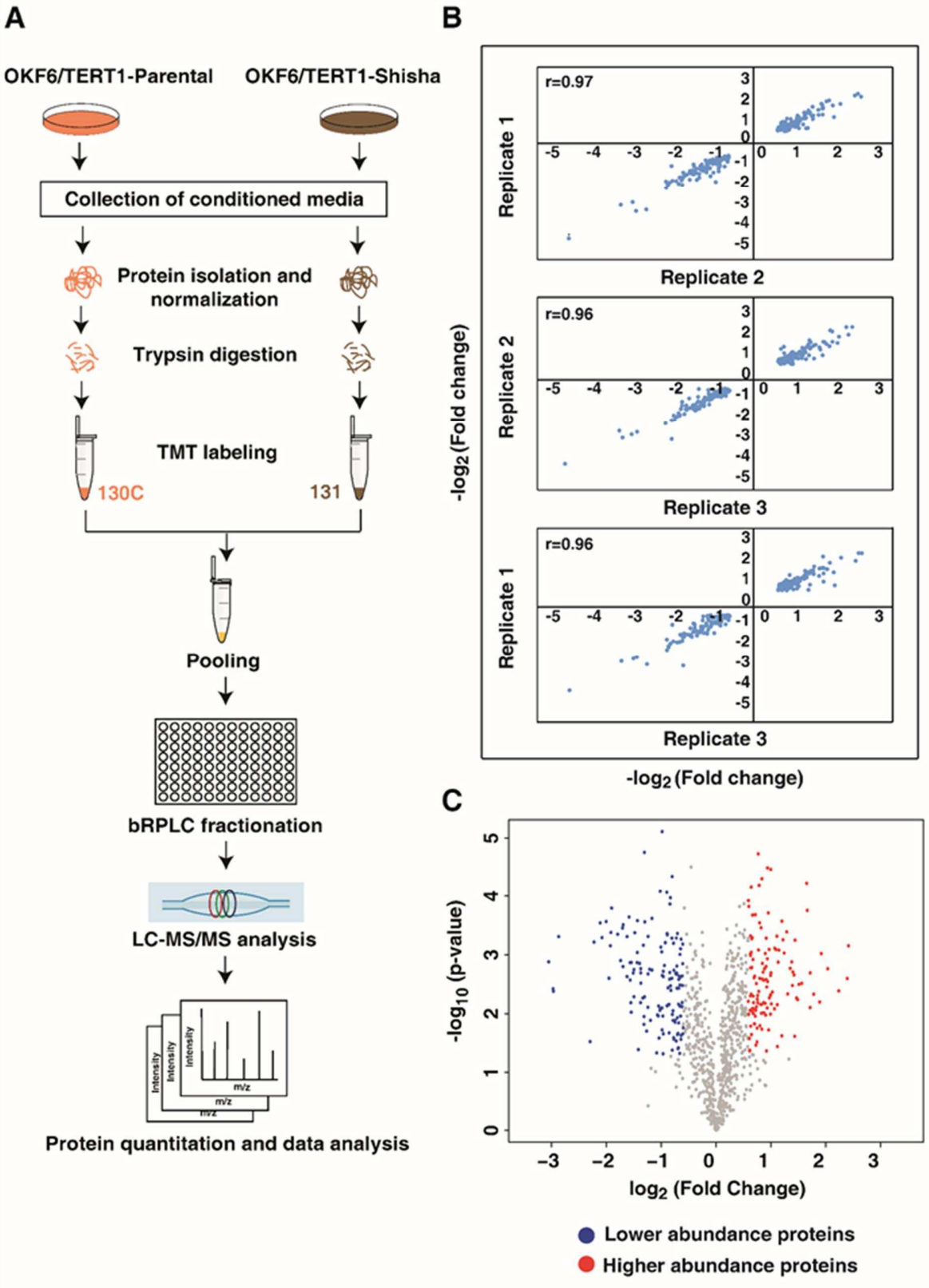

A
OKF6/TERT1-Parental
OKF6/TERT1-Shisha
Collection of conditioned media
Protein isolation and normalization
Trypsin digestion
TMT labeling
130C
131
Pooling
bRPLC fractionation
LC-MS/MS analysis
Intensity
m/z
Protein quantitation and data analysis
B
r=0.97
r=0.96
r=0.96
Replicate 1
Replicate 2
Replicate 3
-log2 (Fold change)
C
-log10 (p-value)
log2 (Fold Change)
Lower abundance proteins
Higher abundance proteins

Figure 2

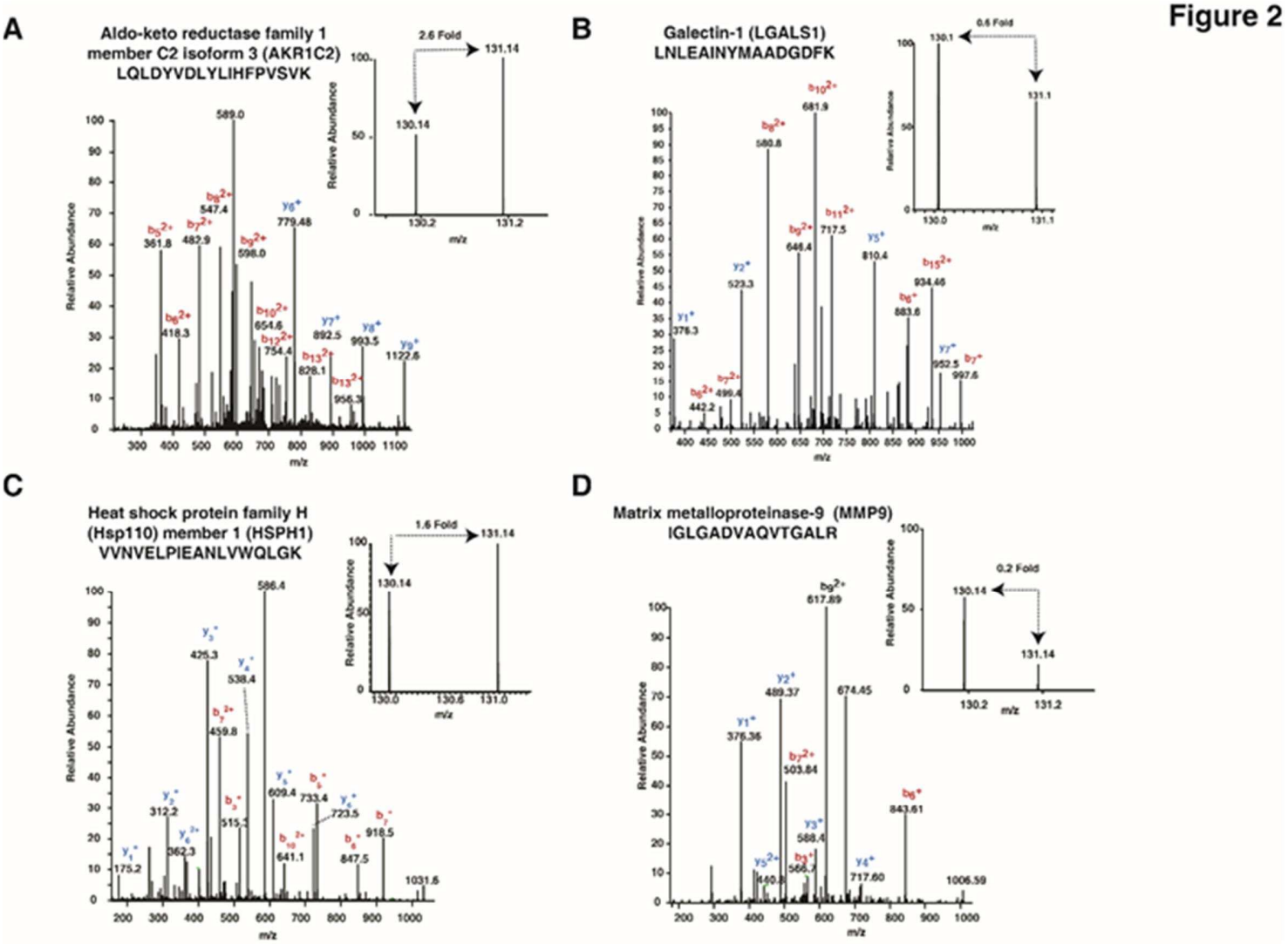
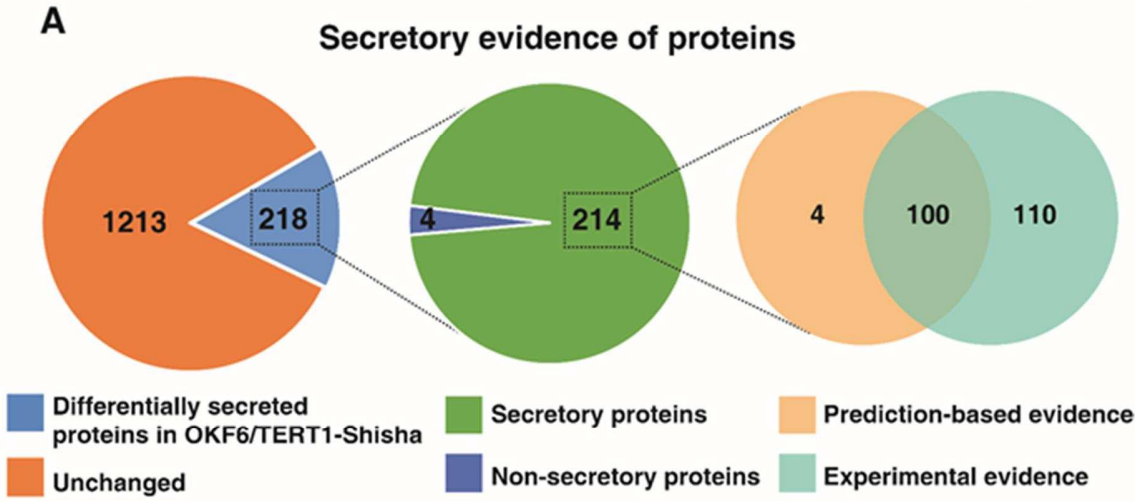
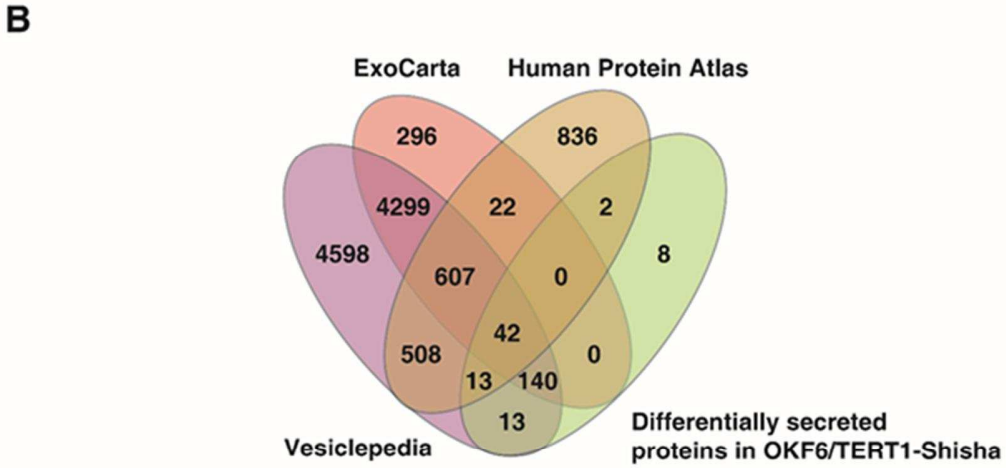
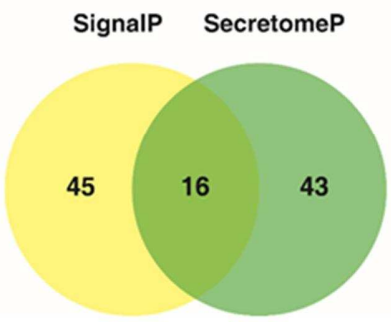

A
Aldo-keto reductase family 1 member C2 isoform 3 (AKR1C2)
LQLDYVDLYLIHFPVSVK
2.6 Fold
131.14
130.14
Relative Abundance
m/z
B
Galectin-1 (LGALS1)
LNLEAINYMAADGDFK
0.6 Fold
130.1
131.1
C
Heat shock protein family H (Hsp110) member 1 (HSPH1)
VVNVELPIEANLVWQLGK
1.6 Fold
131.14
130.14
D
Matrix metalloproteinase-9 (MMP9)
IGLGADVAQVTGALR
0.2 Fold
130.14
131.14

Figure 3


A
Secretory evidence of proteins
1213
218
4
214
4
100
110
Differentially secreted proteins in OKF6/TERT1-Shisha
Unchanged
Secretory proteins
Non-secretory proteins
Prediction-based evidence
Experimental evidence


B
ExoCarta
Human Protein Atlas
296
836
4299
22
2
4598
607
0
8
42
508
0
13
140
13
Vesiclepedia
Differentially secreted proteins in OKF6/TERT1-Shisha


C
SignalP
SecretomeP
45
16
43

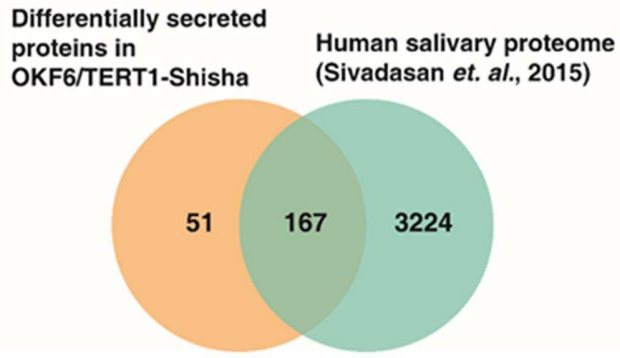
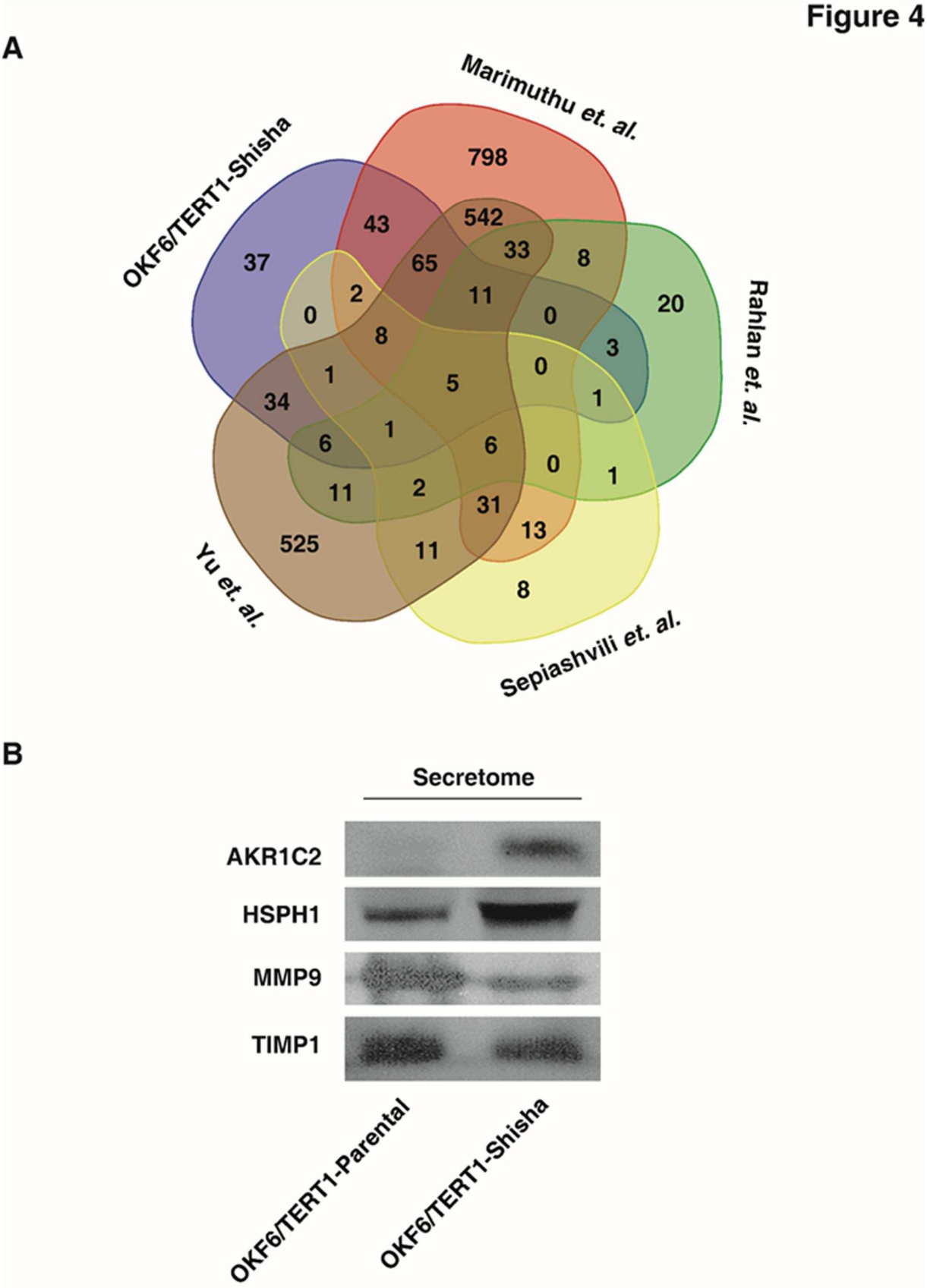

D
Differentially secreted proteins in OKF6/TERT1-Shisha
Human salivary proteome (Sivadasan et. al., 2015)
51
167
3224

Figure 4


A
Marimuthu et. al.
OKF6/TERT1-Shisha
Rahlan et. al.
Yu et. al.
Sepiashvili et. al.
798
43
542
37
65
33
8
2
11
0
20
0
8
3
1
5
0
34
1
6
6
0
1
11
2
31
13
525
11
8
B
Secretome
AKR1C2
HSPH1
MMP9
TIMP1
OKF6/TERT1-Parental
OKF6/TERT1-Shisha