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ORIGINAL PAPER

Whole genome expression profiling in chewing-tobacco-associated oral cancers: a pilot study

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Abstract The current study was undertaken with a view to identify differential biomarkers in chewing-tobacco-associated oral cancer tissues in patients of Indian ethnicity. The gene expression profile was analyzed in oral cancer tissues as compared to clinically normal oral buccal mucosa. We examined 30 oral cancer tissues and 27 normal oral tissues with 16 paired samples from contralateral site of the patient and 14 unpaired samples from different oral cancer patients, for whole genome expression using high-throughput IlluminaSentrix Human Ref-8 v2 Expression BeadChip array. The cDNA microarray analysis identified 425 differentially expressed genes with >1.5-fold expression in the oral cancer tissues as compared to normal tissues in the oral cancer patients. Overexpression of 255 genes and downregulation of 170 genes ($p < 0.01$) were observed. Further, a minimum twofold overexpression was observed in 32 genes and downregulation in 12 genes, in 30–83 % of oral cancer patients. Biological pathway analysis using Kyoto Encyclopedia of Genes and Genome Pathway database revealed that the differentially regulated genes were associated with critical biological functions. The biological functions and representative deregulated genes include cell proliferation (AIM2, FAP, TNFSF13B, TMPRSS11A); signal transduction (FOLR2, MME, HTR3B); invasion and metastasis (SPP1, TNFAIP6, EPHB6); differentiation (CLEC4A, ELF5); angiogenesis (CXCL1); apoptosis (GLIPR1, WISP1, DAPL1); and immune responses (CD300A, IFIT2, TREM2); and metabolism (NNMT; ALDH3A1). Besides, several of the genes have been differentially expressed in human cancers including oral cancer. Our data indicated differentially expressed genes in oral cancer tissues and may identify prognostic and therapeutic biomarkers in oral cancers, postvalidation in larger numbers and varied population samples.

Keywords Oral cancer · Indian patients · Chewing-tobacco habit · Gene expression profile · High-throughput assay · Illumina microarray system

Introduction

Oral cancer is the thirteenth most common cancer in the world, accounting for an estimated 300,373 new cases and 145,238 deaths globally [1]. In India, oral cancer is a major public health problem, ranked as the most common cancer in males with an annual incidence of 53,842 and mortality of 36,436, and the fourth most common cancer in females, with the annual incidence as 23,161 and mortality of 15,631 [1]. The high incidence of oral cancer in India is primarily attributed to the prevalent chewing-tobacco habit, with initiation of the habit at an early age of 9–12 years [2, 3]. Besides, a majority of the Indian patients are diagnosed in advanced stages of the cancer, and the 5-year survival of the oral cancer patients is about 40 % [4]. Despite easy

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accessibility of the sites of oral cancer, presence of preceding precancerous lesions such as oral leukoplakia, erythroplakia and submucous fibrosis in several geographic regions, advances in treatment modalities of surgery, radiotherapy, chemotherapy and targeted therapy such as epidermal growth factor receptor antagonists, prognosis for the cancer is poor. Besides, overall survival of oral cancer patients has not shown a significant improvement in the past four decades. Further, an alarming trend of increased incidence in oral cavity cancers in young adults of 20–45 years, and 60 % increase in the number of oral cancers in <40 years of age in tongue cancer cases, has been reported in the past 25 years [5–7]. Hence, it is imperative to understand the biology and biological behavior of the malignant oral cells. Besides, the overexpression of a panel of biomarkers in oral cancer tissues as compared to clinically normal tissues in the oral cancer patients may enable identification of specific therapeutic targets in oral cancer.

In the past two decades, molecular basis of oral cancer has been extensively investigated [2, 8–11]. Oral intraepithelial neoplasia development occurs by clonal evolution, a stochastic multi-step process with several genetic events contributing to the malignancy, followed by clonal expansion and metastasis [11, 12]. The pathways leading to the cancer are complex, including deregulation of oncogenes, tumor suppressor, DNA damage repair genes, genes associated with signal transduction, metastasis and angiogenesis [11, 13]. While trends can be determined, there is no specific alteration[s] that exists in a majority of the oral cancer patients. It is therefore important to anticipate inherent heterogeneity in oral cancer cell genotype, phenotype and pathogenetic events, as well as host genotype. The differences between individual patients and cancer phenotype including aggressiveness of the disease, response to treatment, recurrence and survival of the patient may be reflected in the expression profile of the patients.

Advances in biotechnology and information technology have facilitated differential expression profiling of the genome of oral cancer, reported primarily from patients in western countries [14–19]. However, there is a dearth of information from the chewing-tobacco-related oral cancer in the Indian patients. In the current pilot study, high-throughput Illumina microarray platform was used to examine differentially expressed genes in oral cancer tissues as compared to clinically normal oral tissues.

Materials and methods

Study subjects

The study included 30 untreated patients with histopathologically confirmed oral squamous cell carcinoma, admitted to Department of Ear-Nose-Throat, Seth G.S. Medical College and King Edward Memorial Hospital, Mumbai, India. Oral cancer biopsy from the non-necrotic central portion of the resected tissues was collected from the patients at surgery. A majority of the patients were habitual long-term tobacco chewers of a minimum duration of 10 years. Three oral cancer patients with chewing-tobacco habit of more than 10 years had stopped chewing-tobacco for 2 months postdiagnosis of the cancer. The patient details are given in Table 1. Briefly, the patient group comprised of 19 males and 11 females, age range of 28–65 years with mean age of 49 years. The primary sites of oral cancer were buccal mucosa—11 cases; tongue—11 cases; gingiva—7 cases; and a single case of lower alveolus. The tumor size of the patients at diagnosis was T1—5 cases, T2—14 cases, T3—8 cases and T4—3 cases. Histopathologically positive lymph node involvement was observed in 18 patients, and 12 patients were node negative. As per TNM classification, the patients were categorized as early stages I and II—9 cases and advanced stages III and IV—21 cases. Clinically, normal oral tissues from contralateral site were available from 27 oral cancer patients. Paired cancer and normal tissues were available from 16 patients. Whereas 14 tissue samples were non-paired with cancer tissues and normal oral samples from different oral cancer patients, RNA from either one of the samples was degraded and showed low RNA integrity number [RIN]. Clinical appearance of leukoplakia, erythroplakia or melanoplakia was not observed in the patients at diagnosis of oral cancer. The tissues were stored in 1 ml RNAlater solution [Ambion Inc, Texas, USA], overnight at 4 °C, and stored at −80 °C until RNA isolation.

Informed consent was obtained from the participants for voluntary participation in the study, and the project was approved by Institute Ethics Committee.

RNA isolation

Total RNA was isolated from tissue samples using RNeasy Mini Kit as per the manufacturer's instructions [Qiagen, Hilden, Germany]. The extracted RNA was quantitated on NanoDrop Spectrophotometer ND-1000 [NanoDrop, Delaware, USA]. The quality and quantity of total RNA were characterized using RNA Nanochip 6,000 kit on Bioanalyzer 2,100 [Agilent Technologies, Foster City, California, USA]. The RNA samples with 18S and 28S ribosomal peaks and RIN between 7 and 10 were used in the microarray experiments.

Illumina microarray assay protocol

Total RNA from cancer tissues and clinically normal tissues was used for microarray analysis assays as per the

Table 1 Clinicopathological data of oral cancer patients

Number	Patient ID	Age	Sex	Tobacco habit	Site	TNM	Stage
1	OC02	35	M	Yes	BM	T1N0M0	I
2	OC03	50	M	Yes[a]	BM	T2N0M0	II
3	OC07	30	F	Yes	BM	T2N0M0	II
4	OC09	60	F	Yes[a]	BM	T2N0M0	II
5	OC15	51	M	Yes	BM	T3N0M0	III
6	OC16	55	M	No	BM	T1N1M0	III
7	OC17	58	F	Yes	BM	T1N1M0	III
8	OC22	50	M	Yes[a]	BM	T1N1M0	III
9	OC23	45	F	Yes	BM	T3N1M0	III
10	OC26	51	M	Yes	BM	T3N1M0	III
11	OC27	55	M	Yes[a]	BM	T4N0M0	IV
12	OC01	60	F	Yes	T	T1N0M0	I
13	OC04	63	M	No	T	T2N0M0	II
14	OC05	30	M	Yes	T	T2N0M0	II
15	OC06	40	M	Yes	T	T2N0M0	II
16	OC11	37	M	Yes[a]	T	T3N1M0	III
17	OC13	60	M	Yes	T	T2N1M0	III
18	OC14	38	F	Yes	T	T2N1M0	III
19	OC21	28	M	Yes	T	T2N1M0	III
20	OC24	65	F	Yes[a]	T	T3N1M0	III
21	OC25	60	F	Yes	T	T2N1M0	III
22	OC29	30	M	No	T	T2N2M0	IV
23	OC08	45	F	Yes	G	T2N0M0	II
24	OC10	62	M	Yes	G	T2N1M0	III
25	OC12	52	M	Yes	G	T2N1M0	III
26	OC19	60	M	Yes	G	T3N1M0	III
27	OC18	54	M	Yes	G	T3N0M0	III
28	OC20	47	F	Yes	G	T3N1M0	III
29	OC28	30	F	Yes[a]	G	T4N1M0	IV
30	OC30	65	M	Yes[a]	LA	T4N1M0	IV

F female, *M* male, *BM* buccal mucosa, *T* tongue, *G* gingiva, *LA* lower alveolus

Yes: Patients were long-term tobacco chewers of minimum 10-year habit

Yes[a]: Eight patients with long-term chewing-tobacco had discontinued chewing-tobacco 2–3 months postoral cancer diagnosis

manufacturer's instructions. Briefly, total RNA was reverse transcribed to cDNA using high-capacity cDNA archive kit [Applied Biosystems, California, USA]. The cDNA was subjected to in vitro transcription in the presence of biotinylated nucleotides [Ambion Inc., Texas, USA]. The biotin-labeled cRNA was fragmented and hybridized to high-density oligonucleotide IlluminaSentrix Human Ref-8 v2 Expression BeadChip arrays [Illumina Inc., San Diego, USA]. The microarray slide contained 22,184 probes representing curated human genes and ESTs. The arrays were scanned using confocal laser scanner, Bead Array Reader and analyzed using GenomeStudio software [Illumina Inc., San Diego, USA].

Microarray data analysis

The intensity output files were initially analyzed for probe hybridization quality control parameters including average background, target intensity and raw noise values. The non-normalized fluorescent intensity of each probe on the chip was obtained using the DirectHyb gene expression package GenomeStudio software [Illumina Inc., San Diego, USA]. The raw data were subjected to average normalization and filtered to select probes with a detection $p < 0.01$ in both cancer tissues and normal buccal mucosa. Differential gene expression analysis was performed using Illumina Custom algorithm. Genes were considered differentially expressed with a 1.5-fold increase or decrease of the transcripts in the tumor tissues as compared to the normal tissues [$p < 0.05$].

Hierarchical clustering analyses were performed on the expression data set using Pearson's correlation to determine distance metric and visualized using Tree View program to group genes according to their similarities in expression levels using an unsupervised clustering algorithm-based software [20] in the tumor [$n = 30$] and control samples [$n = 27$].

The differentially regulated genes were functionally annotated with respect to bioprocesses, molecular function and cellular localization using the Gene Ontology [GO] database [21] and Data Annotation, Visualization and Integrated Discovery [DAVID] [22] bioinformatics tools. The significant gene clusters queried with the known components of biological pathways on the Kyoto Encyclopedia of Genomes and Genes [KEGG] database [23] were used to identify important pathways involved in disease development.

Results

Microarray analysis

The microarray analysis demonstrated >1.5-fold differential gene expression in 425 genes [524 probes] in oral cancer tissues [$n = 30$] as compared to clinically normal oral tissues [$n = 27$], with 255 genes upregulated [Supplement data S1] and 170 genes downregulated [Supplement data S2]. The associated biological functions of the genes [25, 26] are indicated in the Supplement data S1 and S2, and the percentage distribution of the genes as per the biological functions in the various groups synopsized in Table 2. Thus, we observed upregulated genes distributed across various functions such as immune response—22 %, cell metabolism—20.4 %, signal transduction—11 %, cell proliferation—9 %, invasion—8.6 %, cell development—6 %, transcription factors—5.5 %, apoptosis—5.5 %, transport proteins—4.3 %. Two genes [<1 %] belonged to angiogenesis and xenobiotic metabolism group, and functions were unknown for 13 genes [5 %] [Table 2]. The downregulated genes were functionally categorized as cell metabolism—25 %, signal transduction—12 %, cell regulation—10 %, transport proteins—9 %, cell development—8 %, apoptosis—5 %, invasion—3.5 %, transcription factors—3 %, cell proliferation—3 %. Four [2 %] genes each were categorized as immune response and xenobiotic metabolism. The functions were unknown for 16 % genes [Table 2].

Thirty-two genes with twofold overexpression and downregulation in 12 genes are observed and indicated in Tables 3 and 4, respectively. Chromosome location, fold change, percent oral cancer patients with overexpression or downregulation and the biological functions of the genes are also indicated in Tables 3 and 4. A majority [21/32, 65.6 %] of the genes were over expressed in 50–77 % of the cancer samples, with fewer genes showing upregulation in 30 to <50 % cancer samples [Table 3], whereas twofold or more downregulation was observed in 57–83 % of oral cancer samples [Table 4].

The biological pathways and molecular networks were analyzed using DAVID bioinformatic tool with gene enrichment method [22]. A high enrichment score of >12 was obtained for the differentially expressed genes. These upregulated genes were functionally categorized into immune response [12, 37.5 %], signal transduction [2, 6.3 %], cell cycle and proliferation [7, 22 %], invasion and metastasis [4, 12.5 %], apoptosis [2, 6.3 %], cell differentiation [1, 3 %], angiogenesis [1, 3 %], transport [1, 3 %] and xenobiotic metabolizing enzymes [1, 3 %], whereas the 12 genes downregulated were functionally associated with cell metabolism [4, 33 %], cell differentiation [2, 17 %], signal transduction [1, 8 %], invasion and metastasis [1, 8 %], xenobiotic metabolizing enzymes [1, 8 %], cell cycle and cell proliferation [1, 8 %], cell differentiation [2, 17 %], and apoptosis [1, 8 %]. The function of a single gene remained unknown.

Table 2 Biological functions of differentially regulated genes (≥1.5-fold) in oral cancers

Biological function	Genes (%)
Total upregulated genes	$n = 255$
Cell cycle and cell proliferation	23 (9)
Cell differentiation	3 (1.2)
Cell development	15 (5.9)
Cell metabolism	52 (20.4)
Xenobiotic metabolizing enzymes	2 (0.8)
Transport proteins	11 (4.3)
Immune response	56 (22)
Signal transduction	28 (10.9)
Transcription factors	14 (5.5)
Invasion and metastasis	22 (8.6)
Angiogenesis	2 (0.8)
Apoptosis	14 (5.5)
Function unknown	13 (5.1)
Total downregulated genes	$n = 170$
Cell cycle and cell proliferation	5 (2.9)
Cell differentiation	5 (2.92)
Cell development	13 (7.6)
Cell metabolism	42 (24.7)
Xenobiotic metabolizing enzymes	3 (1.75)
Transport proteins	15 (8.7)
Regulatory proteins	17 (9.94)
Immune response	4 (1.75)
Signal transduction	20 (11.7)
Transcription factors	5 (2.9)
Invasion and metastasis	6 (3.5)
Apoptosis	8 (4.7)
Function unknown	28 (16.3)

[a] Differential expression was observed in oral cancer ($n = 30$) as compared to clinically normal tissue ($n = 27$) samples in oral cancer patients

Table 3 Overexpression of genes (≥2-fold) in oral cancer[a]

Gene symbol	Gene	Chromosome number	Fold change	% cancer samples n (%)	Biological function
Cell cycle and cell proliferation					
AIM2	Absent in melanoma 2	1	2.0	15 (50)	Regulation of cell proliferation and reversion of tumorigenesis
APOBEC3B	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	22	2.25	15 (50)	RNA editing enzyme; associated with cell growth and cell cycle control
CCR1	Chemokine (c–c motif) receptor 1	3	3.58	11 (36.7)	Responsible for affecting stem cell proliferation; recruitment of immune cells
FAP	Fibroblast activation protein	2	2.56	12 (40)	Control of fibroblast growth, epithelial—mesenchymal interactions during epithelia carcinogenesis; upregulated in breast and colorectal cancer and wound healing
IL24	Interleukin-24	1	2.82	15 (50)	Controls cell survival and proliferation by inducing STAT proteins
SCHIP1	Schwannomin interacting protein 1	3	2.0	18 (60)	Subunit of eukaryotic initial factor 3 (eIF3c); role in axon function; and inhibition of cell proliferation
TNFSF13B	Tumor necrosis factor(ligand) superfamily, member 13 b	13	2.28	17 (56.7)	Positive regulation of cell proliferation; overexpressed in glioblastoma, high-grade serous carcinoma
Cell differentiation					
CLEC4A	C-type lectin domain, family 4, member A	12	2.51	14 (46.7)	Modulation of differentiation and/or maturation of dendritic cells (DC); involved via its ITIM motif in the inhibition of B cell-receptor-mediated calcium mobilization and protein tyrosine phosphorylation
Transport proteins					
SLC15A3	Solute carrier family 15, member 3	1	2.99	16 (53)	Proton oligopeptide cotransporter; transport-free histidine and certain di- and tripeptides
Xenobiotic metabolizing enzymes					
NNMT	Nicotinamide N-methyl transferase	11	2.39	16 (53)	N-methylation of drugs and other xenobiotic compounds; role in cellular invasion via activating PI3 K/Akt/SP1/MMP-2 pathway in clear cell renal cell carcinoma
Immune response					
C3AR1	Complement component 3A receptor 1	12	2.6	12 (40)	Associated with immune responses
CCL18	Chemokine ligand 18	18	2.67	12 (40)	Chemotactic activity; associated with humoral and cell-mediated immune responses
CD300A	CD300a antigen	17	3.15	16 (53)	Inhibitory receptor; downregulates cytolytic activity in natural killer (NK) cells; mast cell degranulation
CD80	CD80 molecule	3	2.13	12 (40)	Induces T cell proliferation and cytokine production
CD163	CD163 antigen	12	2.86	14 (46.7)	Scavenger receptor for hemoglobin–haptoglobin complex; associated with inflammation.
FCER1G	Fc fragment of IgE, high affinity 1:gamma polypeptide	1	2.32	16 (53)	Associated with allergic responses
GNLY	Granulysin	2	2.05	17 (56.7)	Antimicrobial protein that kills intracellular pathogens; upregulated in Barrett's adenocarcinoma, acute promyelocytic leukemia, chronic lymphocytic leukemia
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	10	2.29	17 (56.7)	Interferon-induced antiviral protein; inhibits expression of viral messenger RNAs; upregulated in OSCC
LILRB3	Leukocyte Ig-like receptor, subfamily b	19	2.7	17 (56.7)	Transduces a negative signal that inhibits stimulation of an immune response. Downregulates antigen-induced B cell activation by recruiting phosphatases to its immunoreceptor tyrosine-based inhibitor motifs

Table 3 continued

Gene symbol	Gene	Chromosome number	Fold change	% cancer samples n (%)	Biological function
MS4A7	Membrane-spanning 4-domains, subfamily A, member 7	11	2.36	18 (60)	Associated with cellular function in the monocytic lineage
SAA1	Serum amyloid a1	11	2.62	14 (46.7)	Major acute-phase reactant; cell adhesion regulator; macrophage chemotaxis
SERPINA1	Serpin peptidase inhibitor, clade A, member 1	11	2.35	16 (53)	Codes for alpha-1 antitrypsin, a serine protease inhibitor (serpin), inhibits other enzymes, including neutrophil elastase released from white blood cells to fight infection
TREM2	Triggering receptor expressed on myeloid cells	6	2.1	19 (63.3)	Involved in chronic inflammation by triggering production of constitutive inflammatory cytokines
Signal transduction					
FOLR2	Folate receptor 2	11	2.19	14 (46.7)	Transport of methotrexate in synovial macrophages in rheumatoid arthritis
MME	Membrane metallo-endopeptidase	3	3.04	9 (30)	Associated with cell–cell signaling; protease inactivates various peptide hormones
Invasion and metastasis					
CTHRC1	Collagen triple helix repeat containing 1	8	2.91	14 (46.7)	Negative regulator of collagen matrix deposition; associated with cancer tissue invasion and metastasis; upregulated in human solid cancers
EMR2	egf-like module containing, mucin-like, hormone receptor-like 2	19	2.18	17 (56.7)	Member of adhesion-GPCR receptor family; expressed predominantly by cells of the immune system; associated with cell migration
SPP1	Osteopontin (multiple functions)	4	3.61	16 (53)	Increased expression observed in esophageal cancer, ovarian and breast cancer; expression correlated with enhanced metastasis in hepatocellular carcinoma
TNFAIP6	Tumor necrosis factor, a-induced protein 6	2	2.97	16 (53)	Member of the hyaluronan-binding protein family; involved in extracellular matrix stability and cell migration, cell–cell and cell–matrix interactions during inflammation and tumorigenesis
Angiogenesis					
CXCL1	Chemokine (C-X-C) motif ligand 1	4	2.47	17 (56.7)	Involved in angiogenesis, inflammation, tumorigenesis and wound healing
Apoptosis					
GLIPR1	Glioma pathogenesis-related protein 1	12	2.22	23 (76.7)	Proapoptotic activities; decreased expression in prostate and bladder cancer cells
WISP1	Wnt1 inducible signaling pathway protein 1	8	2.77	16 (53)	Associated with cell survival; attenuates p53-mediated apoptosis in response to DNA damage through activation of AKT kinase; upregulates antiapoptotic Bcl-X(L) protein

[a] Upregulation was observed in oral cancer ($n = 30$) as compared to clinically normal tissue ($n = 27$) samples in oral cancer patients

Hierarchical clustering analysis

Clustering analysis of genes was performed with GenomeStudio software [Illumina]. Heat map of unsupervised cluster analysis of cancer samples [$n = 30$] and clinically normal samples [$n = 27$] defined three clusters [Fig. 1]. Data visualization tool using color grid representing various degrees of gene expression delineated a cluster of 14 samples with a pattern of specific genes downregulated; a distinct second cluster of 15 samples with specific upregulated genes; and a third cluster of 29 samples with the combination of both upregulated and downregulated genes [Fig. 1]. A single sample data were deleted from further analysis due to incomplete clinicopathological information. We observed clustering of five tongue tissues and five buccal mucosa samples from both cancer patients and normal controls. Six of the 16 paired cancer and clinically normal samples demonstrated near-identical gene expression profiles.

Discussion

The human genome project decoding the genome and the concurrent advances in biotechnology and information

Table 4 Downregulated genes (≥2-fold) in oral cancer[a]

Gene symbol	Gene	Chromosome number	Fold change	% cancer samples n (%)	Biological function
Cell cycle and cell proliferation					
TMPRSS11A	Transmembrane protease, serine 11A	4	−2.13	23 (76.7)	Role in cellular senescence; overexpression inhibits cell growth and induce G1 cell cycle arrest; associated with esophageal and oral squamous cell carcinoma, and breast cancer
Cell differentiation					
CALML5	Calmodulin-like 5	10	−2.0	21 (70)	Calcium-binding protein; expressed in the epidermis and related to the calmodulin family of calcium-binding proteins; overexpression detected in reconstructed epidermis and is restricted to differentiating keratinocytes
ELF5	E74-like factor 5	11	−2.46	23 (76.7)	Transcriptional regulation of differentiation of keratinocytes
Cell metabolism					
ALDH3A1	Aldehyde dehydrogenase 3 family, member A1	17	−2.06	20 (66.7)	Detoxification of alcohol-derived acetaldehyde; involved in metabolism of corticosteroids, biogenic amines, neurotransmitters and lipid peroxidation
BCMO1	Beta-carotene 12, 15-monooxygenase	16	−2.79	24 (80)	Retinoic acid biosynthesis
GATM	Glycine amidinotransferase	15	−2.21	23 (76.7)	Catalyzes biosynthesis of guanidinoacetate, immediate precursor of creatine
GDPD3	Glycerophosphodiester phosphodiesterase domain containing 3	16	−2.0	17 (56.7)	Glycerol metabolic process
Xenobiotic metabolizing enzymes					
CYP4F12	Cytochrome P450, family 4, subfamily F, polypeptide 12	19	−2.16	22 (73)	Monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids
Signal transduction					
HTR3B	5-Hydroxytryptamine receptor 3B	11	−2.12	25 (83)	Receptor for serotonin; a ligand gated ion channel, when activated causes depolarizing responses
Invasion and metastasis					
EPHB6	EPH receptor B6	7	−2.0	22 (73)	Transmembrane protein that functions as a receptor for ephrin-B family proteins; does not contain a functional kinase domain; influences cell adhesion and migration; expression downregulated during tumor progression, suggesting that the protein may suppress tumor invasion and metastasis; associated with lung, breast and colorectal cancer, melanoma, astrocytoma, glioblastoma and leukemia
Apoptosis					
DAPL1	Death-associated protein-like 1	2	−2.14	23 (76.7)	Mediating interferon-gamma-induced cell death; associated with early stage of epithelial differentiation
Function unknown					
ANKRD35	Ankyrin repeat domain 35	1	−2.0	20 (66.7)	Unknown function

[a] Downregulation was observed in oral cancer ($n = 30$) as compared to clinically normal tissue ($n = 27$) samples in oral cancer patients

technology provided the basic infrastructure for high-throughput genome-wide associations and expression analysis for understanding the biological processes in human cancers. In the current pilot study, toward examining biomarkers associated with frankly malignant tissues in the oral cavity, we investigated expression of genes in oral cancer tissues as compared to normal tissues from oral cancer patients. We observed >1.5-fold differential regulation in 425 genes in oral cancer tissues as compared to paired and unpaired control normal buccal mucosa from oral cancer patients. Further, a twofold overexpression was observed in 32 genes in 30–77 % and downregulation in 12

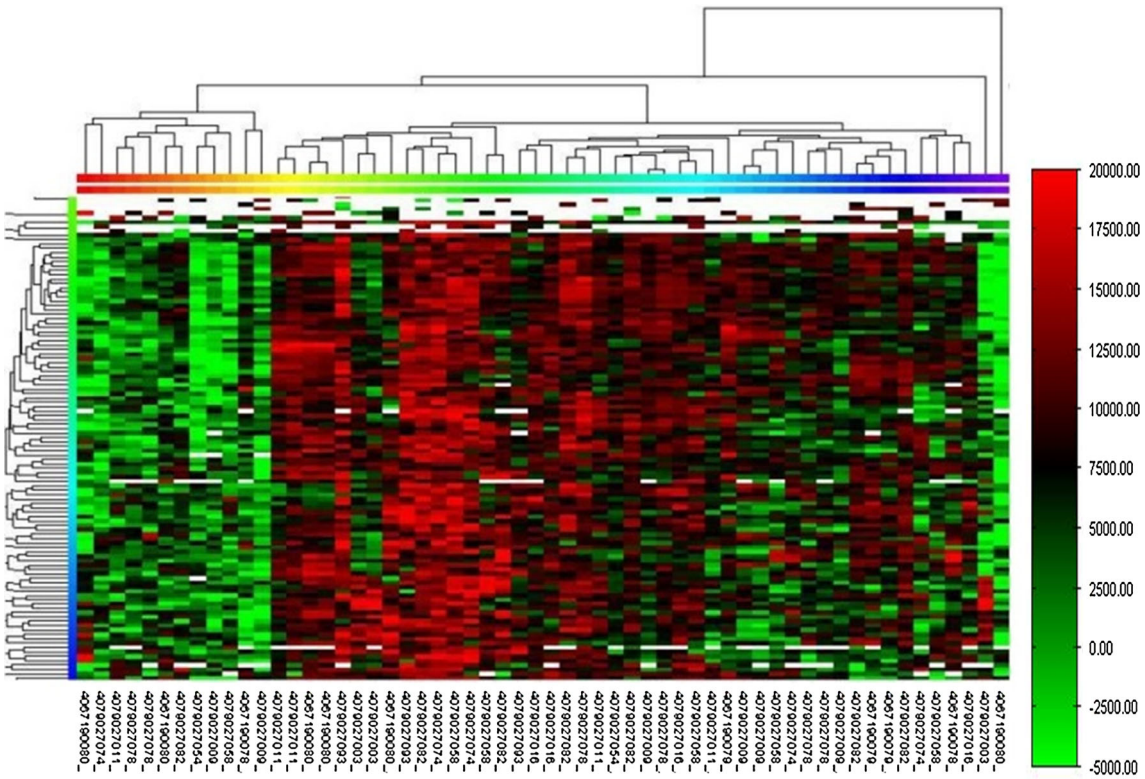

Fig. 1 Heat map generated by hierarchical clustering of differentially expressed representative genes between oral cancer and control samples. The rows represent genes, and the columns are samples. Hierarchical clustering of gene expression data for representative genes in 58 samples (oral cancer samples = 30 and control samples = 27; a single oral cancer sample was excluded from further analysis due to insufficient clinical information. The *color grid* indicating expression levels is shown in *right-hand side bar*. The expression analysis shows four clusters with, cluster 1 comprising 12 samples in the *left panel*, and 2 samples on extreme *right panel*, representing 14 normal samples; cluster 2 and 3 include two closely related clusters of 15 + 14 oral cancer samples; cluster 4 comprising 13 apparently normal clinical samples and 2 oral cancers

genes in 57–83 % oral cancer tissue samples. A significant association of the differentially expressed genes with clinicopathological features e.g., site, lymph node, metastasis and cancer stage was not observed due to smaller sample numbers in the stratified subgroups. We observed a near-identical gene expression profile in 6 of 16 paired cancer and clinically normal samples, indicating that the clinically normal tissues may contain initiated malignant cells due to the process of field cancerization indicating high risk of conversion to a malignant phenotype.

Biological pathway analysis of the differentially expressed genes in oral cancer, analyzed through KEGG pathway [23], demonstrated association with critical biological pathways. Genes associated with cell metabolism and signal transduction comprised 31 % of the upregulated genes and 37 % of the downregulated genes, indicating a critical role for these genes in oral carcinogenesis. The immune response and cell cycle/cell proliferation genes were upregulated in 31 % of the oral cancer samples and may result in deregulation of the genes. The functions of 20 % of the differentially expressed genes are not known.

A diagrammatic representation of the upregulated genes in cell survival, proliferation, migration, angiogenesis and apoptosis is indicated in Fig. 2. Thus, tumor necrosis factor (ligand) superfamily, member 13 b (TNFSF13b), binds to its receptor leading to the activation of transcription factors BCL and BFL for cell survival [25], whereas interleukin-24 (IL-24), tumor necrosis factor a-induced protein 6 (TNFAIP6), chemokine (c–c motif) receptor 1 (CCR1) and absent in melanoma 2 (AIM2) may lead to enhanced cell proliferation via activating the JAK-STAT, MAPK and AKT pathways [24]. SPP1/osteopontin overexpressed 3.6-fold in oral cancer tissues has been associated with wound healing, inflammation, immune response, bone remodeling and tumorigenesis [26]. Several transcription factors including PPRA via GLIPR1 and a combination of TCF, Ep300 and β-catenin activate Wnt1 inducible signaling pathway protein 1 (WISP1) results in the deregulation of apoptosis [25]. Further, invasion and metastasis are mediated through egf-like module containing mucin-like hormone receptor-like 2 (EMR2) and collagen triple helix repeat containing 1 (CTHRC1) [25]. Overexpression of

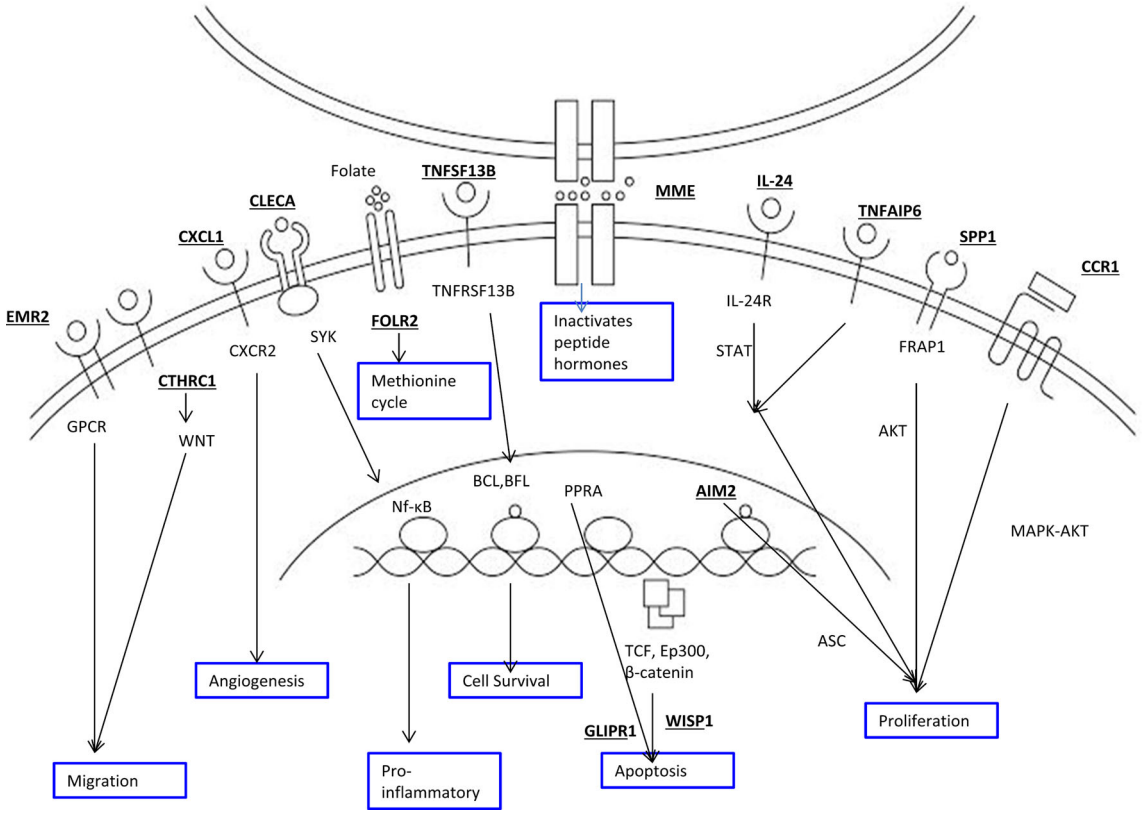

Fig. 2 Upregulated genes associated with cellular pathways including cell proliferation, migration, angiogenesis, apoptosis and inflammatory immune response

chemokine motif ligand 1 (CXCL1) binds to CXCR2 and results in angiogenesis, whereas C-type lectin domain, family 4 A (CLEC4A), activates the NF-kβ pathway [25] (Fig. 2). Hence, it is feasible that overexpression of the various genes and deregulation of various cellular pathways may lead to oral cancer.

Besides, the dominant role of overexpression of the mentioned genes, several genes associated with cell regulation, protein transport and cell development were downregulated in 26 % of cancers, indicating disruption in the functions. Death-associated protein-like 1 (DAPL1) deregulation leads to decreased caspase activation which decreases apoptosis [24, 25]. Aldehyde dehydrogenase 3 family, member A1 (ALDH3A1) and cytochrome P450, family 4, subfamily F, polypeptide 12 (CYPF12) are associated with detoxification and deregulation leading to increased toxicity [25]. Downregulation and decreased expression of glycine amidinotransferase (GATM) and beta-carotene 12, 15-monooxygenase (BCMO1) associated with cell metabolism may alter the biological pathways promoting cancer development or progression [24, 25].

Several differentially expressed genes have been earlier demonstrated in oral squamous cell carcinomas. Thus, IFIT2 upregulated 2.3-fold in 57 % of our oral cancer samples is in concordance with Lai and co-workers reporting enhanced expression of IFIT2 in oral cancers as compared to matched non-cancerous oral tissues in Taiwanese group of patients [26]. On the other hand, IFIT2 downregulation has been associated with increased invasiveness and epithelial to mesenchymal transition [27]. The chemokine receptor CCR1 upregulated 3.58-fold in 37 % in our study mediates dual role of directional migration and local host defense against tumor in oral cancer [28] and hepatocellular cancers suggesting multiple functions of this gene in tumorigenesis [29].

The genes overexpressed in our oral cancer tissues have been associated with several additional human cancers. FAP and AIM2 promoted invasive phenotype in colorectal cancer [30–32], and FAP and SPP1 were overexpressed in breast cancer [33–35]; SPP1 was also associated with poor prognosis in lung cancer [36]; NMMT in invasive renal cell carcinoma [37]; TNFSF13B in glioblastoma [38]; CTHRC1 in esophageal cancers [39]; and CXCL1 in melanoma [40]. On the other hand, TMPRSS11A downregulated in the cancer tissues in our study has been associated with breast cancer [41]; EPHB6, a metastasis suppressor gene, is downregulated in non-small cell lung cancers [42].

The current pilot study using high-throughput microarray analysis to differentiate the clinically normal buccal mucosa and frankly malignant oral cancers may indicate biomarkers of predictive, prognostic and treatment response. The expression analysis should be validated in larger sample/control sizes in various geographic regions via alternative technology such as real-time PCR. The identification of the deregulated genes and the cellular pathways may provide potential new treatment targets, understanding of the mechanistic pathways and insight into oral cancer development with transformation and progression of normal cells to a malignant phenotype.

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Conflict of interest No potential conflicts of interest are disclosed.

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