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Review

Recent trends of saliva omics biomarkers for the diagnosis and treatment of oral cancer

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ABSTRACT

Background: Recent advances in surgery, radiotherapy, and chemotherapy have no significant effect on oral cancer survival rates due to late diagnosis, poor tumor response to chemotherapy and radiotherapy, as well as a lack of effective biomarkers for early diagnosis.
Highlights: Therefore, an investigative study aimed at identifying genomics, proteomics, metagenomics, and, metabolomics derived biomarkers for early diagnosis may improve the survival rate of oral cancer patients. Identification and application of saliva-based "omics" biomarkers may overcome painful invasive procedures currently being used for the diagnosis of oral cancer. One single biomarker may not be able to differentiate between oral squamous cell carcinoma (OSCC) and controls. Thus, multiple sensitive and specific biomarkers may be needed for screening high-risk patients and following them up for early signs of OSCC occurrence. Validation of these biomarkers in large patient cohorts is, however, required before they can be used in clinical practice.
Conclusion: In this review, we summarize the potential of omics derived salivary biomarkers as diagnostic and prognostic tools in oral cancer detection and the future clinical benefits associated with these markers.

Contents

1. Introduction

Oral cancer is prevalent where there is a significant use of tobacco, betel quid, or alcohol. In India, oral cancer is more prevalent among males who indulge in tobacco chewing and

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smoking [1]. Leukoplakia, erythroplakia, and oral submucous fibrosis are the premalignant lesions associated with oral squamous cell carcinoma (OSCC). Advances in surgery, radiotherapy, and chemotherapy have had no significant effect on oral cancer mortality rates due to the absence of early diagnostic biomarkers and poor response to treatment [2]. Presently, diagnosis and monitoring of OSCC requires invasive procedures, such as the endoscopic collection of biopsy specimens and repeated drawing of blood for pathological diagnosis. The location in the mouth from where the biopsy sample is drawn is crucial for the histopathological verification of oral cancer. However, selecting the proper location is difficult due to the non-uniform appearance of cancerous and precancerous lesions [3].

Therefore, in order to improve survival rates of oral cancer patients, identification of biomarkers required for early diagnosis is essential. Identification and application of saliva-based genomics, proteomics, metagenomics, and metabolomics derived biomarkers may overcome painful invasive procedures. Accumulation of both genetic and epigenetic changes, which lead to a gain of function in certain oncogenes and a loss of function in some tumor suppressor genes of oral carcinogenesis, are well documented. ‘‘Omics’’ biomarkers in saliva, such as metabolites, proteins, transcribed genes, miRNAs, genome alterations, and epigenomic changes, as well as the microbiome have been proposed for use in the diagnosis of oral cancer [4,5]. In this review, we summarize the role of salivary biomarkers as diagnostic and predictive tools for oral cancer.

2. Biochemical properties of saliva

Human saliva contains water (99%), proteins (0.3%), and inorganic substances (0.2%). The pH of saliva, which is slightly acidic in nature, is in the range of 6.0 to 7.0. Daily, 1–1.5 L of serous and mucinous saliva is produced by the acinus cells of the salivary glands (parotid, submandibular, and sublingual glands) and it is subsequently released into the oral cavity [6]. The pH of the saliva of a healthy individual is 7.4. Whole Saliva (WS) can be collected via “unstimulated” or “stimulated” methods. Stimulated saliva is produced by the parotid gland, whereas unstimulated (resting) saliva is produced by the submandibular gland. Stimulation of saliva production decreases the concentration of small molecules, such as myoglobin, and alters the total composition of the saliva in favor of larger molecules. Therefore, unstimulated saliva may preferable for biomarker discovery and diagnostic studies. Tumors within the oral cavity may shed cellular material directly into saliva [7].

3. Advantage of Saliva over other non-invasive biomarkers

Saliva samples are safe to handle. Bio-molecules in saliva are known to inhibit the human immunodeficiency virus. Hence, the chances of transmission from saliva are very low as compared to transmission from blood samples. Compared with blood samples, saliva samples are easy to store, as saliva does not clot [8]. Non-invasive saliva procurement is painless and can be done by anyone. This dramatically reduces anxiety and discomfort of patients. Cell free saliva (CFS) contains thousands of proteins as well as mRNA and microRNA (miRNA) transcripts, and metabolites which have wide range of biological functions [9]. Blood DNA which is derived from a diverse population of immune cells is dependent on individual states of immune activation, whereas salivary DNA is derived from a homogenous subpopulation of polymorphonucleated leukocytes and epithelial cells of the oral cavity. Saliva is produced and collected locally from sites close to the oral cavity tumors which enables the detection and analysis of tumor-specific biomolecules with minimal interference. Flow rates, age, drug use, sex, diet, physiological status and salivary gland pathology may affect analyte concentrations present in saliva [10].

4. Genetic polymorphism of GSTT1 in salivary DNA

GSTT1 is involved in the detoxification of active metabolites of tobacco carcinogens considered the most important causative factor of OSCC. A GSTT1 null genotype was reported in 24% of oral cancer patients and 16% of controls in Salivary DNA [1].

5. Role of circulating tumor DNA (ct DNA) in saliva for oral cancer diagnosis and treatment

Early stage OSCC patients (stage I or II) are treated via surgery or radiation alone. A combination of surgery, chemotherapy, and radiation therapy is applied to treat advanced-stage disease (stages III and IVA/B). Metastatic disease (stage IV C) is treated with combination chemotherapy or single agent chemotherapy depending on the patient's response to therapy. Molecular characterization of OSCC tumors is delayed due to the heterogeneous nature of this tumour. This has a negative impact on patient prognosis and survival. Circulating tumor DNA (ctDNA), ($<$ 1.0% of total Cell free DNA: cfDNA), is shed into body fluids from both primary tumors and metastatic lesions. This ctDNA provides a genetic fingerprint of the tumor, where its level can be used to monitor the tumor burden in post-treatment situations. Tumors at advanced stages of the diseases have higher levels of ctDNA, whereas those at early-stages of the disease have lower levels. The ctDNA levels rapidly increase with disease progression and decrease following successful therapeutic intervention. Tumor DNA was detected in the saliva of a 100% of oral cancer patients, and in the plasma of 80% of patients with oral cancers. Thus saliva is preferentially enriched with tumor DNA. Liao et al., reported mutations in exon 4, codon 63 of p53, in the saliva of OSCC patients. Techniques used to detect ctDNA in cancer patients include: droplet digital polymerase chain reactions (ddPCR); beads, emulsion, amplification, magnetics (BEAMing); pyrophosphorolysis-activated polymerization (PAP); and Safe Sequencing System (SafeSeqS) [11].

6. Salivary methylome signatures

In promoter hypermethylation, methyl groups are transferred to cytosine residues in the CpG islands. The methyl group silences the gene. Methylation has been suggested to be an early event in OSCC. Promoter hypermethylation is a more frequent cause of gene silencing than genetic mutation [12,13]. In OSCC, hypermethylated genes, recognized as potential biomarkers for oral cancer, are associated with cell proliferation, DNA repair, apoptosis, cell-cell adhesion, and angiogenesis [14]. Aggressive tumors with poor prognosis and a greater burden of epigenetic alteration may be frequently detected in the saliva of OSCC patients. The prognostic significance of hypermethylation in the saliva of OSCC patients before treatment is related to a higher frequency of methylated signaling in exfoliated cells. Aggressive tumors with a higher burden of epigenetic alteration are more frequently detected in salivary rinses [15–18].

Hyper methylated genes such as CCNA1 (Cell Cycle Regulator), DAPK1 (Programmed cell death), DCC (Receptor for nectrin required for axon Guidance), EDNRB (G-protein-coupled receptor),

Table 1
Deregulated salivary mRNAs and their association with OSCC.

Gene Symbol	Gene Name	Chromosome location	Expression Profile in saliva	Functional Importance in Cancer
DUSP1	Dual specificity protein phosphatase 1	5q35.1	Up	Responsible for Chemoresistance and radioresistant properties in solid tumors
H3F3A	H3 Histone Family 3A	1q41	Up	Cell Proliferation
ILIB	Interleukin 1 Beta	2q14.1	Up	Inflammation, cell proliferation, differentiation, and apoptosis
IL8	Interleukin 8	2q35	Up	Tumor angiogenesis, cell adhesion, immunity, and cell cycle arrest
SAT1	Spermidine/Spermine N1-Acetyl-transferase 1	Xp22.11	Up	Cell Proliferation and angiogenesis
OAZ1	ornithine decarboxylase antizyme 1	19p13.3	Up	Cell growth and proliferation by controlling intracellular levels of polyamine.
S100 P	S100 calcium binding protein P	4p16	Up	Cell cycle regulation and differentiation
MMP-1	Matrix Metallopeptidase 1	11q22.2	Up	Associated with the progression of dysplasia to cancer through enhancement of susceptibility to angiogenesis.
MMP-9	Matrix Metallopeptidase 9	20q13.12	Up	Associated with the progression of dysplasia to cancer through enhancement of susceptibility to angiogenesis.

KIF1A (Protein kinase involved in apoptosis and DNA damage response), MINT31 (Calcium channel regulator), p16INK2A (Receptor of sonic hedgehog), RASSF1α (Induced growth inhibition along the RAS-activated signaling pathway), and TIMP3 (T-cell antigen receptor, recognition of foreign antigens) are commonly reported in the saliva of OSCC patients [12,15].

MGMT, *p16*, *DAPK*, and *RASSF1* were frequently methylated in paired saliva and tissue samples of OSCC patients [19–22]. LINE-1 hypomethylation has been reported in salivary rinses obtained from OSCC patients, compared with healthy controls [15]. Aberrant methylation of ECAD, RARβ, FHIT, p15, TIMP3, and APC has been reported in the saliva samples of oral cancer patients [19]. TIMP3 inhibits tumor growth, angiogenesis, invasion, and metastasis [20]. Pattani et al.; reported that hypermethylation of Endothelin receptor type B (EDNRB) promoter in saliva was associated with an increased risk for oral cancer and pre-malignancy [23]. Promoter methylation of *EDNRB* in saliva of oral cancer patients may be useful for risk assessment [24]. Hypermethylation of the putative tumor-suppressor gene, DCC (deleted in colorectal cancer), has been detected in the saliva of OSCC patients. DCC is located at 18q21 and encodes a transmembrane protein. DCC is involved in both epithelial and neuronal cell differentiation [25].

7. Salivary transcriptome signatures

The salivary transcriptome is defined as a pool of RNA species found in saliva. Molecular profiling of mRNA in saliva provides information regarding gene transcription in both normal and OSCC individuals. Long ncRNA, small nucleolar RNA and miRNA have been detected in saliva. Alterations of mRNAs and miRNAs have been reported in OSCC [26,27].

Salivary mRNA and miRNA are present in apoptotic bodies and are actively released in exosomes or microvesicles [28]. Exosomes protect salivary miRNA from extracellular ribonucleases. The miRNAs in saliva control cell proliferation, differentiation, apoptosis, and immune function via the regulation of target genes. Native salivary miRNAs are significantly more stable in saliva than exogenous miRNAs. Ago 2 is a protein component of the RNA-induced silencing complex, and it is found in supernatant saliva. Ago 2 is responsible for salivary miRNA stability [26,29].

The salivary miRNAome may be useful for biomarker discovery due to its role in epigenetic modulation of cellular adaptation over time. Down regulation of tumor suppressor genes by over expression of certain miRNAs and up-regulation of oncogenes by under-expression of certain miRNAs are responsible for oral carcinogenesis. The miRNAs may act as either tumor suppressors or oncogenes [30,31]. The miRNAs are located at fragile sites or cancer-associated genomic regions [30]. Therefore, salivary miRNAs may be used as early diagnostic biomarkers for oral cancer screening [32].

The validity of cancer related components of its transcriptome as diagnostic and prognostic markers of tumorigenesis supports the unique potential of saliva. Salivary RNAs are protected from degradation by exosomes, which are membrane bound cell organelles with a diameter of 30–100 nm and originate in the endoplasmic reticulum. Profiling of mRNAs and miRNAs in exosomes provides insight into the diagnosis of oral cancer. Salivary miRNAs as well as salivary mRNAs may be used as potential diagnostic markers for oral cancer [33].

Based on microarray analysis, Li et al., reported significant overexpression of dual specificity protein phosphatase 1 (DUSP1), H3 Histone Family 3A (H3F3A), Interleukin 1 Beta (ILIB), Interleukin 8 (IL8), ornithine decarboxylase antizyme 1 (OAZ1), Spermidine/Spermine N1-Acetyltransferase 1(SAT), and the S100 calcium binding protein P (S100 P) in the saliva of OSSC patients. These saliva mRNAs may be used a panel for oral cancer detection [34–36] (Table 1).

DUSP1 activates the MAPK pathway, which participates in protein modification, oxidative stress, and signal transduction [8]. Hypermethylation of the DUSP1 gene has been reported to be associated with oral carcinogenesis [36]. *IL-IB* is involved in inflammation, cell proliferation, differentiation, and apoptosis. *IL8* is a proinflammatory cytokine and a neutrophil chemotactic factor, which plays a key role in tumor angiogenesis, cell adhesion, immunity, and cell cycle arrest [8]. St. John et al., have reported that IL-8 expression in saliva, at both mRNA and protein levels, may hold promise as a biomarker for OSCC [37]. H3F3A acts as a proliferative marker for oral cancer. S100 P is responsible for cell cycle regulation and differentiation. *MMP-1* and *MMP-9* transcripts have been found to be over-expressed in the saliva of OSCC patients, compared with that of controls [38–40] and have been associated with the progression of dysplasia to cancer via enhancement of susceptibility to angiogenesis [41].

Due to significant over expression in comparison to mRNAs, miRNAs show as potential biomarkers in the diagnosis of oral cancer. These miRNAs are particularly useful for the characterization of poorly differentiated OSCC. Park et al., reported that expression levels of miR-125a and miR-200a were significantly reduced in the saliva of oral cancer patients as compared to healthy controls [29]. MiR-125a plays an important role in cell proliferation and *miR-200a* is involved in tumor suppression and early metastasis [29,42]. Liu et al., reported that miR-31 expression was higher in the saliva of OSCC patients as compared to blood, indicating local release directly from the tumor site. Levels of salivary miR-31 in OSCC patients declined significantly following

surgical ablation of their tumors. This implies that salivary miR-31 may be derived directly from tumor tissue. Significant over-expression of salivary miR-31 was reported in all clinical stages of oral cancer, irrespective of tumor size, compared to that of healthy controls [41]. Tumor suppressor miR-31 is absent in metastatic oral tumors. Therefore, salivary miR-31 may have potential application in the monitoring or detection of residual or recurrent OSCC [41].

Analysis of genome-wide expression patterns of salivary miRNAs using a NanoString and Counter miRNA expression assay and real-time quantitative PCR (qPCR), indicated that 11 out of 700 tested miRNAs were significantly down regulated (miR-136, miR-147, miR-1250, miR-148a, miR-632, miR-646, miR-668, miR-877, miR-503, miR-220a, miR-323-5p), whereas 2 miRNAs (miR-24, miR-27b) were over-expressed in the saliva of OSCC patients, compared with healthy controls. Down regulation of miR-136 and up regulation of miR-27b was detected in OSCC patients as compared to patients with recurrent tumor. The release of miR-27b into saliva from OSCC cells, therefore, may result in significantly elevated levels of salivary miR-27b. Thus, miR-27b may be a promising salivary-biomarker for OSCC detection [32].

The expression of miR-139-5p in saliva was significantly reduced in the tongues of SCC patients. Significant over expression of miR-10b, miR-660, miR-708 and miR-30e and under expression of miR-145, miR-let-7, miR-99b, miR-181c and miR-197 were reported in the saliva of progressive low grade dysplasia (LGD) patients, as compared to those with non-progressive LGD leukoplakia [43,44]. Most deregulated miRNAs in progressive LGD leukoplakias patients showed the same expression patterns as OSCC. Over-expression of tumor suppressive miRNAs, such as miR-197, miR-let-7, miR-99a/b, miR-126 and miR-145 were noticed in non-progressive LGD leukoplakias, compared with progressive LGD leukoplakias. This implied that non-progressive LGD leukoplakias may possess mechanisms for protection from malignant transformation [43].

A highly significant increase in salivary miR-21 levels in OSCC patients may play a pivotal role in OSCC tumorigenesis and invasiveness via the Wnt/β-catenin pathway by targeting DKK2. MiR-184, which acts as an oncogene by inducing cell proliferation and inhibiting cell apoptosis by targeting c-Myc is significantly increased in OSCC saliva. MiR-21 and miR-184 appear to act synergistically on the AKT pathway [45,46], inducing cell proliferation and inhibiting cell apoptosis. Yang et al., reported that the miR-145 levels in the saliva of OSCC patients were decreased [47]. This miRNA suppresses tumors by decreasing cell proliferation or promoting cell apoptosis by targeting K-RAS [48], Fli1 [49], c- Myc [50], and DFF45 [51]. Down-regulation of c-Myc and Cdk6 (cyclin-dependent kinase 6) expression was regulated by miR-145 [52]. These findings indicate that salivary miR-21, miR-145, and miR-184 may be used as non-invasive, rapid diagnostic biomarkers for malignant transformations that occur in the oral cavity. Salivary miRNA analysis is a promising non-invasive assay for the early detection and monitoring of oral pre-malignancies [53] (Table 2).

The presence of lncRNAs, such as MALAT-1 (Metastasis-associated lung adenocarcinoma transcript 1) and HOTAIR (HOX antisense intergenic RNA) in the saliva of OSCC patients is well-documented. Patients with lymph node metastases showed higher expression levels of *HOTAIR* in their saliva. HOTAIR reduces the expression of E-cadherin by recruiting EZH2 to the promoter region of E-cadherin and H3K27me3 modification in OSCC tumors. Differential expression of HOTAIR has been reported in the saliva of metastatic OSCC patients compared with that of primary cancer controls. High level expression of *MALAT1* in cancer is responsible for cell proliferation, migration and invasion. Screening of differentially expressed lncRNAs in the saliva of a large cohort of OSCC patients may strengthen the usefulness of potential oral cancer biomarkers [54–56].

8. Salivary proteomics signatures

The salivary proteome is defined as the entire protein content of the oral cavity. More than 2000 proteins in Saliva are synthesized and secreted into the oral cavity by salivary gland acinar cells and are involved in maintaining homeostasis of the oral cavity [26,57]. The salivary proteome has been useful for identifying biomarkers for OSCC via advanced proteomic technologies, using mass spectrometry, liquid chromatography, and protein/peptide labelling, which allow the detection of low abundance molecules in the saliva proteome. Several studies have reported that the proteomic profiles of the saliva of OSCC patients and healthy controls are different [9].

Several protein biomarkers have been reported in the saliva of OSCC patients (Table 3). Endothelin-1 was identified as a potential biomarker for OSCC development in patients with oral lichen

Table 2
Commonly deregulated salivary miRNAs and their association with OSCC.

miRNA	miRNA ID	Chromosome location	Expression Profile in saliva	Functional Importance in Cancer
miR-125a	hsa-miR-125a	19q13.41	Down	Inhibits cell proliferation
miR-200a	hsa-miR-200a	1p36.33	Down	Tumor suppression and early metastasis
miR-31	hsa-miR-31	9p21.3	Up	Enhance cell proliferation, promotes metastasis, monitoring or detecting residual or recurrent tumor.
miR-136	hsa-miR-136	14q32.2	Down	Tumor-suppression and promotes apoptosis.
miR-27b	hsa-miR-27b	9q22.32	Up/Down	Inhibition of cell proliferation through inhibiting FZD7 and FZD7-mediated Wnt signaling pathway.
miR-139-5p	has-miR-139	11q13.4	Down	Cell proliferation, apoptosis, lymph node involvement and metastasis, metastatic potential of the tumors, a poor prognosis
miR-197	hsa-miR-197	1p13.3	Up	Cell proliferation
miR-let-7	hsa-miR-let-7	21	Up	Involved in the process of metastasis
miR-99a/b	hsa-miR-99a	21q21.1	Down	Tumor suppression
miR-126	hsa-miR-126	9q34.3	Down	Tumor suppression
miR-145	hsa-miR-145	5q32	Down	Decreased proliferation, or promoted apoptosis by targeting K-RAS, c- Myc and DFF45
miR- 21	hsa-miR-21	17q23.1	Up	Tumorigenesis and invasiveness through the Wnt/β-catenin pathway, by targeting DKK2
miR-184	hsa-miR-184	15q25.1	Up	Inducing proliferation and inhibiting apoptosis by targeting c-Myc
miR-181c	hsa-miR-181c	19p13.12	Down	Tumor Suppressor
miR-10b	hsa-miR-10b	2q31.1	Up	Cell proliferation
miR-708	hsa-miR-708	11q14.1	Up	Induce carcinogenicity by down-regulating Caspase-2 level.
miR-24	hsa-miR-24	9q22.32	Up	Enhances proliferation and reduces apoptosis

Table 3
Deregulated salivary proteins and their association with OSCC.

Protein Symbol	Protein Name	Chromosome location	Expression Profile in saliva	Functional Importance in Cancer
EDN1	Endothelin-1	6p24.1	Up	Promote tumorigenesis
IL-8	Interleukin-8	4q13.3	Up	Recruitment and activation of neutrophils, basophils, and T-cells which are in inflammatory response.
IL-1β	Interleukin 1 Beta	2q14.1	Up	Inflammatory response, cell proliferation, differentiation, and cell survival.
M2BP	Mac-2-binding protein	17q25.3	Up	Modulation of cell-cell and cell-matrix interactions
CD59	CD59 Molecule	11p13	Up	Activation of T cells.
MRP14	S100 Calcium Binding Protein A9	1q21.3	Up	Recruitment, adhesion and migration of leukocytes. Induction of cytokine and chemokine secretion.
CCND1	Cyclin D1	11q13.3	Up	Cell proliferation and metastasis.
Ki67	Marker Of Proliferation Ki-67	10q26.2	Up	Cell proliferation and metastasis.
LDH	Lactate Dehydrogenase	11p15.1	Up	Production of lactate from pyruvate under anaerobic conditions which is key feature of cancer cells.
MMP-9	Matrix Metallopeptidase 9	20q13.12	Up	Migration, chronic inflammation, angiogenesis and metastasis.
OGG1	8-Oxoguanine DNA Glycosylase	3p25.3	Down	Repair of oxidative DNA damage
SERPINB5	Maspin or Serpin Family B Member 5	18q21.33	Down	Inhibition of tumor angiogenesis
MMP 2	Matrix Metallopeptidase 2	16q12.2	Up	Regulation of vascularization and metastasis
IL-6	Interleukin 6	7p15.3	Up	Down regulation of apoptosis and induction of uncontrolled cell proliferation thorough inactivation of p53 tumor suppressor gene by hypermethylation of its promoter region
TNF-α	Tumor Necrosis Factor-Alpha	6p21.33	Up	Stimulation of cell proliferation, inhibition of apoptosis and secretion of proinflammatory cytokines through transcriptional activation of NF-kB
IL-1α	Interleukin 1 Alpha	2q14.1	Up	inflammation and angiogenesis
VEGF-A	Vascular Endothelial Growth Factor A	6p21.1	Up	Angiogenesis, endothelial cell proliferation and migration.
bFGF	Fibroblast Growth Factor 2 (Basic)	4q28.1	Up	Endothelial cell proliferation, invasion, and angiogenesis progression

planus [58]. *IL-8* [39], *IL-1β, glycoprotein M2BP* (Mac-2-binding protein), *CD59,* myeloidrelated protein 14 (*MRP14)* and catalase have been identified as salivary biomarkers of oral cancer [1]. Shiptzer et al., reported that levels of Cyclin D1, Ki67, lactate dehydrogenase (LDH) and matrix metalloproteinase (MMP)-9 were elevated, whereas levels of 8-oxoquanine DNA glycosylase (OGG1) and Maspin were reduced in the saliva of oral cancer patients [59]. Matrix metalloproteinases (MMP2 & MMP9) are involved in the degradation of the extracellular matrix and basement membranes. This induces the release of VEGF, which enhances angiogenesis. MMPs are associated with tumor invasion and metastasis [60].

Brailo et al., reported that levels of IL-6 and the tumor necrosis factor alpha (TNF-α) were significantly higher in the saliva of oral leukoplakia patients. IL-6 down regulates apoptosis and induces uncontrolled cell proliferation via inactivation of the p53 tumor suppressor gene by hypermethylating its promoter. TNFα stimulates cell proliferation, inhibits apoptosis and enhances secretion of proinflammatory cytokines through transcriptional activation of NF-kB [61]. Rhodus et al., reported that IL-1, IL-6, IL-8 and TNF-α levels were significantly higher in the saliva of oral cancer patients as compared to those of oral dysplasia patients [62]. The levels of 5 cytokines (IL-1α, IL-6, IL-8, VEGF-A, and TNF-α) were significantly higher in the saliva of tongue squamous cell carcinoma patients as compared to controls [63]. Cytokines are known to be involved in inflammation and angiogenesis.

Zhong et al., reported that telomerase was overexpressed in the saliva of oral cancer patients [64]. De Jong et al., reported that levels of the cytoskeletal proteins, actin and myosin, were consistently higher in the saliva of oral cancer patients compared to those in patients with premalignant lesions [65]. Actin and myosin facilitate cell motility and invasion. Salivary transferrin is also reported as an early detection biomarker for oral cancer [65]. Increase in salivary transferrin is correlated with increasing tumor size. Transferrin is involved in cancer cell proliferation and survival [66]. Tumor markers such as Cyfra 21-1, tissue polypetide antigen [TPA] and cancer antigen CA-125 were significantly over expressed in the saliva of OSCC patients [67].

Basic fibroblast growth factor (bFGF) levels were found to be significantly over expressed in the saliva of OSCC patients as compared to healthy subjects. Significant over expression of bFGF has been observed in the saliva of newly diagnosed OSCC patients, as compared to those in remission as well as oral leucoplakia patients. Salivary bFGF may be used as a potential biomarker for detecting recurrence in post-treatment OSCC patients. Fibroblast growth factors (FGFs) are reportedly involved in endothelial cell proliferation, invasion, angiogenesis and progression of OSCC. In the salivary glands, bFGF is present in the parasympathetic nerve and in ductal epithelial cells. Elevated salivary bFGF levels found in newly diagnosed OSCC patients may have originated from salivary glands, neoplastic cells and tumor-associated angiogenesis [68,69].

9. Salivary microbiome signatures

Significant microbial populations which colonize oral cavities are referred to as the salivary microbiome. Most bacteria found in saliva are attached to exfoliated human epithelial cells. The oral microbiome level varies between saliva and subsites of the oral cavity such as the buccal mucosa, supragingival and subgingival plaque. *Streptococci,* which are the dominant bacteria, co-exist with *Veillonella, Gemella, Rothia*, *Fusobacterium*, and *Neisseria* species. Use of tobacco is one of the most important risk factors for oral cancer. Studies have established that tobacco smoking affects the composition of the salivary microbiome profile in both smoking and non-smoking of healthy individuals as well as oral cancer patients. Oral microbiota may affect the oral cancer risk due to local induction by tobacco, alcohol and betel quid-related carcinogens that directly affect tumor growth by activating tumor cell toll-like receptors (TLR), leading to that eventual cytokine production. Microbes and their metabolomic products can induce

Table 4
Deregulated salivary microbiome and their association with OSCC.

Microbial Phylum	Microbial Genus/Species	Species involved in Molecular pathogenesis of OSCC
Actinobacteria	*Actinomyces (A. dentalis)*	Development of dental plaque and subsequent caries or periodontal diseases.
Firmicutes	*Streptococcus (S.mitis, S.salivarius, S.sanguis, S. pneumonia)*	*Streptococcus* spp.: synthesis of acetaldehyde which interacts directly with lipid peroxidation of the cell and induce DNA damage.
	Peptostreptococcus	Maintenance of acidic and hypoxic microenvironment of tumors by reduced levels of *Lactobacillus* spp.
	Lactobacillus	
	Veilonella,	
	Gemella	
	Eubacterium,	
	Staphylococcus (S.aureus)	
	Selenomonas	*Gemella* species: Involvement of immune-suppression of cancer patients
	Granulicatella	*Eubacterium:* infections of periodontal diseases.
		Staphylococcus aureus affects the host immune system.
		Members of the genus *Selenomonas* associated with chronic and aggressive periodontitis.
Spirochaetes	*Treponema*	Association with the inflammation of periodontal tissues
Proteobacteria	*Aggregatibacter*	*Aggregatibacter* stimulates the expression of proinflammatory cytokines.
	(A. segnis)	*Neisseria spp.* have high alcohol dehydrogenase activity that converts ethanol to acetaldehyde.
	Neisseria	
	(N.flava, N. flavescens, N.elongata),	
	Eikenella	*Eikenella :* association with HPV-negative head and neck squamous cell carcinoma
	Haemophilus	
Fusobacteria	*Leptotrichia (L.buccalis)*	Leptotrichia : production of lactic acid.
	Fusobacterium (F. nucleatum subsp. Polymorphum, F.periodonticum)	*Fusobacterium nucleatum* stimulates tumorigenesis through interaction with Toll-like receptors of oral epithelial cells. Modulation of several antiapoptotic pathways. Activation of p38, resulting in the secretion of MMP-9 and MMP-13 (collagenase 3).
Bacteroidetes	*Porphyromonas. (P. gingivalis)*	*P. gingivalis* prevents apoptosis of gingival epithelial cells. It also promotes invasion of oral cancer through activation of MMP9
	Prevotella (P.oris)	
	Alloprevotella (A.tannerae)	
	Capnocytophaga (C.gingivalis, C. ochracea)	*Prevotella* taxa: potential/opportunistic pathogens. Association with pain and swelling and 'wet' canal of oral diseases

mutations in tumor suppressor genes and proto-oncogenes which control cell proliferation and/or survival, invasion and tumor suppression. Alteration of microenvironments in the oral cavity of normal individuals may change the microbial composition of their saliva. *Streptococci* induce an acidic, hypoxic tumor environment by producing short chain organic acids. Chronic inflammation caused by bacterial infection is responsible for tumorigenesis [70].

Association of salivary microbiome profiles with oral cancer may lead to an understanding of the etiology of oral cancer, thereby potentially opening new research paradigms for OSCC (Table 4). *Prevotella, Veillonella, Porphyromonas and Capnocytophaga species* are elevated in the saliva of oral cancer patients. Increased levels of *Porphyromonas* is associated with immune suppression in OSCC patients. Microbial processes of pathogenic bacteria may be used as a potential diagnostic and prognostic biomarker for OSCC. The abundance of Bacteroidetes (*Prevotella*), Proteobacteria (e.g. *Haemophilus* and *Neisseria*) and Firmicutes (e.g. *Streptococcus* and *Veilonella*) was higher in healthy individuals, whereas the abundance of *Actinomyces* (Actinobacteria), *Schwartzia* (Firmicutes), *Treponema* (Spirochaetes) and *Selenomonas* (Firmicutes) was higher in OSSC patients. *Fusobacteria, Streptococcus, Porphyromonas, Leptotrichia*, and *Aggregatibacter* were the most abundant genera in OSCC. *Prevotella oris, Neisseria flava, N. flavescens/subflava, Fusobacterium nucleatum ss polymorphum, Aggregatibacter segnis, Streptococcus mitis, Fusobacterium periodonticum, Neisseria elongata, Porphyromonas sp. and Alloprevotella tannerae* were the most abundant bacterial species in OSCC. *Fusobacterium nucleatum* induced pro-inflammatory responses in oral epithelial cells by activating several chemokines [4].

Streptococcus salivarius preferentially colonizes saliva compared to teeth, whereas *Streptococcus sanguis* preferentially colonize teeth. *Capnocytophaga gingivalis, C. ochracea, Eubacterium sabureum, Leptotrichia buccalis,* and *Streptococcus mitis* were the most abundant bacterial species in the saliva of OSCC patients. *Gemellaceae or Aggregatibacter* was more abundant in the saliva of normal individuals compared to that of HNSCC patients [71–76]. Previous reports have indicated that *Lactobacillus and Weeksellaceae* were more abundant in the saliva of HPV positive OSCC patients, while *Eikenella, Neisseria, and Leptotrichia* were more abundant in the saliva of HPV negative OSCC patients. Salivary microbiome profiles were significantly different between OSCC patients treated with surgery and OSCC patients treated with chemo-radiation therapy and surgery (CRT/Surgery). *Haemopilus, Neisseria, Aggregatibacter and Leptotrichia* were significantly enriched in patients treated with surgery, while

Table 5
Deregulated salivary metabolites and their association with OSCC.

Name of Metabolites	Level in Saliva of OSCC Patients	Biological Function
Lactic acid	Cancer cells produce excess lactic acid	End product of glycolysis
Polyamines (cadaverine and putrescine)	Increased	Cell division
γ-aminobutyric acid (GABA)	Glutaminolysis increased utilization of GABA	Cell proliferation and inhibition of apoptosis through the activation of the p38 MAPK and the inhibition of the JNK MAPK signaling pathways.
Ornithine	Increased	Induction of tumor cell differentiation
5-hydroxymethyluracil	Elevated due to oxidative stress	Formed when cells are under oxidative stress
Sphinganine and phytosphinganine	Down	Involved in ceramide synthesis which is responsible for inducing apoptosis
Carnitine	Lower because fatty acid metabolism is down-regulated	Differentiation in early and late stage disease in response to the higher energy demand of the tumor. Increased membrane synthesis and cellular turnover.
Glutamic acid	Down-regulated in tumor cells	Cell proliferation and invasion
Succinic acid	Up-regulated	Inhibits prolyl hydroxylase-containing enzymes.

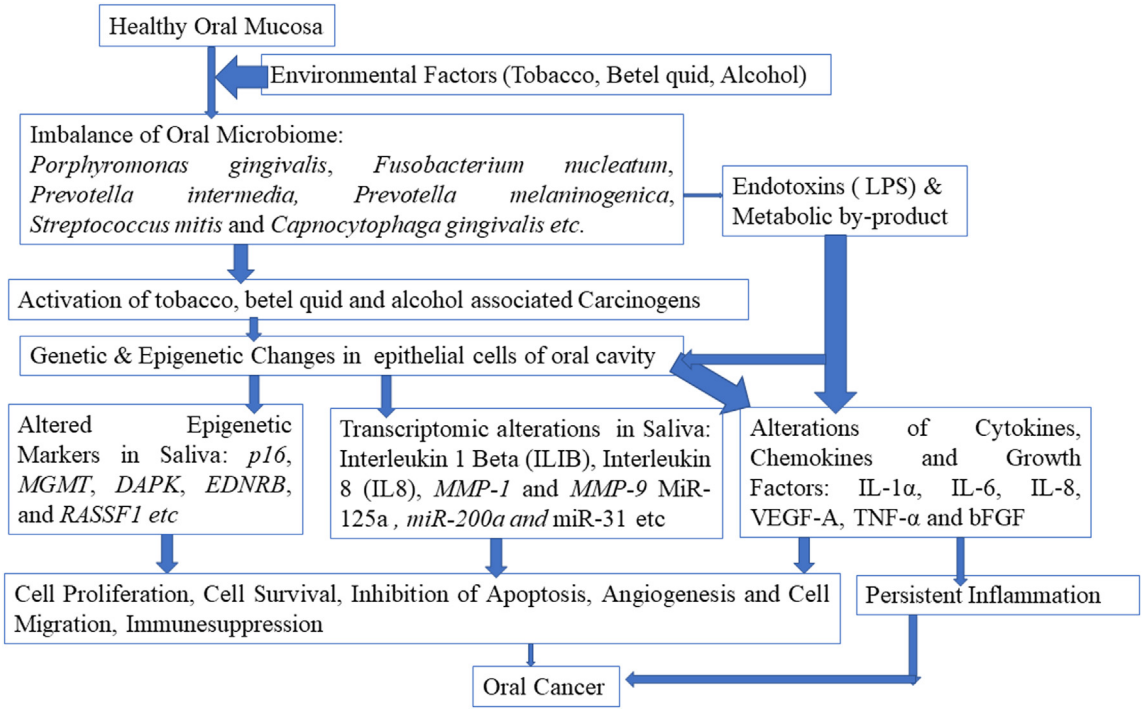

Fig. 1. Schematic over-view of molecular pathogenesis of OSCC.

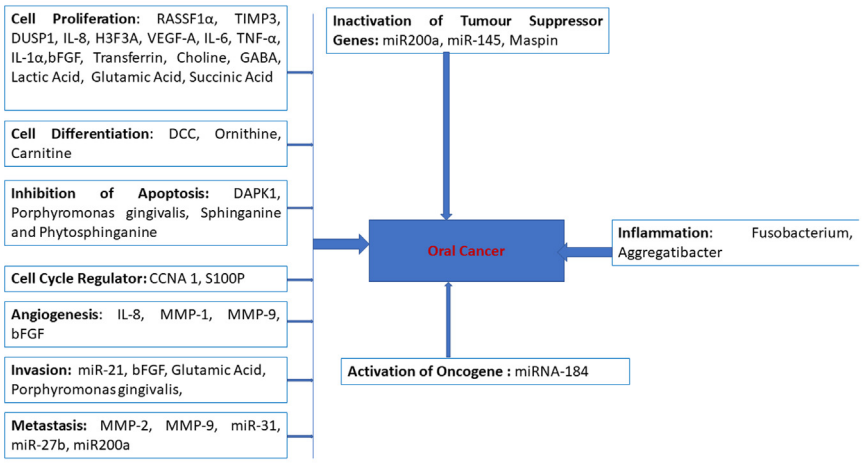

Fig. 2. Salivary genomics, proteomics, metagenomics and metabolomics biomarkers involved in biological processes that drive oral cancer.

Table 6
Summary of salivary omics biomarkers in oral cancer diagnosis and treatment.

Omics Biomarkers in Saliva	Role in Oral Diagnosis and Treatment
Promoter Methylation in EDNRB	Progression of Oral cancer
Over expression of DUSP1, IL-1β, IL-8, OAZ1, and S100P genes at mRNA level	Early detection of oral cancer
Over expression of MMP-1 and MMP-9 at mRNA level	Progression of dysplasia to Oral cancer
Reduced level of miR200a	Indication of early metastasis
Loss of miR-31	Detection of recurrent OSCC
Elevation of miR-27b	Early detection of oral cancer
Overexpression of miR-21 and miR-184 and reduced expression of miR145	Early detection of oral cancer
Increased expression of Transferrin protein	Early detection of oral cancer
Overexpression of bFGF	Detection of recurrent OSCC
Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella melaninogenica, Streptococcus mitis, and Capnocytophaga gingivalis	Diagnostic indicator of OSCC
Up regulation of lactic acid, 5-hydroxymethyl uracil, phenylalanine, valine and phosphocholine	Diagnostic indicator of OSCC

Lactobacillus and Lactobacillaceae showed significant enrichment in patients treated with CRT/Surgery [75].

Cigarette smoke also affects the survival of gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae* in the oral cavity. Actinobacteria and Fusobacteria populations were increased, whereas that of Firmicutes was decreased in smokers. Individuals with a habit of smoking and drinking had decreased Fusobacteria populations in their saliva. The relative abundance of *Streptococcus anginosus* was higher in oral lesions of betel nut chewers. The abundance of *Prevotella and Capnocytophaga* was significantly increased, whereas that of *Granulicatella, Staphylococcus, Peptostreptococcus and Gemella* was significantly decreased in smokers [72].

Functional analysis of Microbiomes revealed that genes of bacteria in the saliva of OSCC patients were mostly involved in the pentose phosphate pathway, porphyrin metabolism, and ascorbate and aldehyde metabolism. Genes of bacteria in the saliva of healthy individuals were mostly associated with lipid and fatty acid biosynthesis, inorganic ion transport and metabolism, glutathione metabolism, citrate cycle/TCA cycle, ubiquinone and other terpenoid quinine biosynthesis pathways and tetracycline biosynthesis [73].

Detection of differences between the salivary microbiomes of OSCC and healthy subjects may be useful in the identification of cancer-associated changes at a given time point; however, evaluation of the relative stability of adult oral microbiomes offers an opportunity to monitor shifts in bacterial communities over time [4].

Porphyromonas gingivalis, *Fusobacterium nucleatum*, and *Prevotella intermedia* are associated with OSCC [71,77]. A higher abundance of *Prevotella melaninogenica, Streptococcus mitis* and *Capnocytophaga gingivalis* in the saliva may also act as diagnostic indicators of OSCC [71]. *P. gingivalis* infection suppressed apoptosis by inducing the expression of B7-H1 and B7-DC receptors in each OSCC cell and primary gingival epithelial cells. B7-H1 expression induces regulatory T cells (Treg) that suppress effector T cells and contribute to immune evasion in OSCC. *P. gingivalis* infection may induce metalloproteinase (proMMP-9) expression by activating the ERK1/2- Ets1, p38/HSP27, and PAR2/NF-KB pathways [78]. F. nucleatum infection results in the secretion of MMP-9 and MMP-13 (collagenase 3) which reportedly contribute to OSCC metastasis [79].

10. Salivary metabolomics signatures

Metabolomics may be defined as the measurement of all intracellular metabolites. It is a potent tool that enables a better understanding of cellular functions. The metabolome is the end product of transcription and translation. The dynamics of primary metabolism operate on a scale of seconds [9]. Metabolomic studies are essential for identifying metabolic alterations related to oral cancer and chemotherapeutic drug exposure [80–84]. Sugimoto et al., compared salivary metabolic profiles between oral cancer patients and healthy controls [80]. Metabolites such as taurine, leucine, isoleucine, pyrroline, choline, cadaverine, tryptophan and valine were found to be the differentiating factor between oral cancer and healthy controls [7] (Table 5).

Wang et al., reported that 8 metabolites, lactic acid, p-hydroxyphenylacetic acid, N-nonanoylglycine, 5-hydroxymethyluracil, succinic acid, ornithine, hexanoylcarnitine and propionylcholine, were significantly up-regulated and 6 metabolites, carnitine, 4-hydroxy-L-glutamic acid, acetylphenylalanine, sphinganine, phytosphingosine, and S-carboxymethyl-L-cysteine, were significantly down regulated in the saliva samples of OSCC patients. High levels of choline, betaine, and pipecolinic acid and low levels of L-carnitine were also reported in OSCC saliva. Tumor cells often produce large amounts of lactic acid via glycolysis due to excessive proliferation. Elevation of 5-hydroxymethyluracil in OSCC patients may be due to cancer related oxidative stress. Sphinganine and phytosphingosine are involved in synthesis and metabolism of ceramide (N-Acylsphingosine)which induces apoptosis [85].

Wei et al., reported the presence of metabolites, such as γ-aminobutyric acid, phenylalanine, valine, n-eicosanoic acid, and lactic acid, in the saliva of oral leukoplakia and OSCC patients. Three salivary metabolites, phenylalanine, valine, and lactic acid, were used to distinguish OSCC patients from healthy controls with a high degree of sensitivity and specificity [82]. An increased level of choline in the saliva of OSCC patients was associated with a high rate of cancer cell proliferation. Choline is rapidly metabolized into phosphocholine and then oxidized to betaine in tumors. Increased levels of phosphocholine and glycerophosphocholine were reported in the saliva of OSCC patients [83].

Alteration of glycosylation is a key feature of tumor cells. Tumor cells secrete glycoconjugates into body fluids. Salivary glycoproteins levels, including those of total sialic acid (TSA) and a-L-fucosidase, were significantly higher in oral cancer patients. Increased levels of 3-hydroxybutyric acid in the saliva of OSCC patients may be associated with a higher metabolic turnover and the demand for membrane biosynthesis to meet cell proliferation requirements, leading to a higher lipid utilization rate [84,85].

11. Conclusion

The application of saliva for diagnostics has limitations due to the lack of correlation between biomolecules of blood and saliva and the circadian variation of salivary biomolecules. In order to

minimize individual biological variation, a combination of different salivary biomarkers was used as a panel to detect oral cancer. Saliva has a broad spectrum of applications related to oral cancer such as risk assessment, prognostic application and the monitoring of response to therapy. Saliva may be used for population-wide screening for disease progression [86,87]. Application of salivary biomarkers will be essential for early diagnosis as well as good prognosis, leading to decreased OSCC mortality rates. The oral microbiome is an ideal source of biomarkers as compared to other tumor biomarker sources, due to low-inter and intra individual variations.

Salivary biomarkers may exist in a variety of different forms, such as microbes, DNA, RNA, lipids, metabolites, and proteins, which are associated with the risk, progression, or chance of recurrence of OSCC (Figs. 1 and 2). Such biomarkers are also helpful in the prognosis, diagnosis, and treatment of oral carcinomas. Potential biomarkers discovered via “omics” techniques such as those involving the proteome, transcriptome, miRNAome, metabolome, microbiome, or epigenome, are required to be validated via a large number of populations studies prior to FDA evaluation and approval. Salivary genetic and epigenetic analyses provide information regarding the host genome, oral microbiota, and DNA virus infection. The salivary epigenome reflects the mechanisms through which gene expression changes over time. Early detection and diagnosis may lead to higher survival rates and play a significant role in the successful clinical treatment of OSCC. One single biomarker may not enable differentiation between OSCC and controls, and multiple sensitive and specific biomarkers may be required for screening high-risk patients and to follow them-up for early signs of OSCC recurrence (Table 6). Validation of these biomarkers and biomarker panels in large patient cohorts is, however, required before they can be used in clinical practice.

CRediT authorship contribution statement

Indranil Chattopadhyay: Writing - original draft, Writing - review & editing*. **Madhusmita Panda:** Writing - original draft, Writing - review & editing.

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