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The emerging landscape of salivary diagnostics

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High-morbidity and high-mortality illnesses, including cancer and cardiovascular, metabolic and neurological diseases, are difficult to diagnose without supplemental laboratory testing. The earlier a disease is detected and diagnosed, the more likely it is that appropriate treatment will be administered, thus reducing the severity of the disease for the patient. Early detection is therefore vital for implementing effective clinical treatment. However, three limitations prevent the full benefits of clinical diagnostics from being realized: definitive biomarkers associated with disease; simple and inexpensive methods that are minimally invasive; and an accurate, portable and easy-to-use diagnostic platform.

Saliva is a biofluid comprising secretions of the salivary glands (the parotid, submandibular, sublingual and other minor salivary glands), oral mucosa cells, blood and gingival crevicular fluid (Fig. 1). Similarly to serum and other biofluids, saliva contains biomolecules such as DNA, mRNA, microRNA, protein, metabolites and microbiota. Because obtaining saliva can be low cost, noninvasive, simple and does not cause patient discomfort, it is a highly desirable body fluid for biomarker development for clinical applications. The aim of this review was to provide a status review of salivary 'omics' constituents, the mechanism of salivary diagnostics and their translational and clinical applications.

Salivaomics

Saliva contains a variety of biomolecules, including DNA, mRNA, microRNA, proteins, metabolites and microbiota; changes in the salivary concentration of these biomolecules can be used to develop dysregulated biomarkers to help identify early oral and systemic diseases, evaluate disease prognosis and risk, and monitor the response to treatment (22, 89). The term 'salivaomics' was coined in 2008 to reflect knowledge about the various 'omics' constituents in saliva, including the genome, epigenome, transcriptome, proteome, metabolome and microbiome (2, 107) (Fig. 2).

The salivary genome and epigenome

The salivary genome consists of both human and microbial DNAs. Nearly 70% of the salivary genome is of human origin, whereas the remaining 30% is from the oral microbiota (81). The quality of salivary DNA is good: 72–96% of samples can be genotyped; 84% can be amplified; and 67% can be sequenced (33, 64) and stored long term without significant degradation (9). Salivary genetic and epigenetic analyses provide gene-transcription profiles that reflect abnormal pathological genetic processes.

DNA methylation is an epigenetic process that can change in response to the passage of time, development or environmental exposure (14). Aberrant methylation of genes (e.g. promoter hypermethylation) is common in cancers (17, 19). Chi et al. (102) completed a methylation array on DNA extracted from preoperative saliva, postoperative saliva and tissue from patients with oral squamous cell carcinoma, as well as on DNA from saliva of healthy control subjects (i.e. without oral squamous cell carcinoma). They found significant differences in DNA-methylation patterns between the preoperative and postoperative saliva of patients with oral squamous cell carcinoma and between preoperative saliva from patients with oral squamous cell carcinoma and

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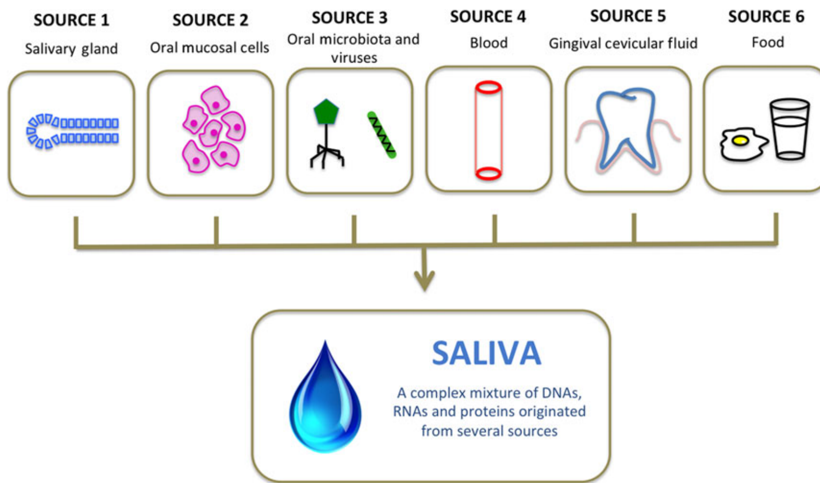

Fig. 1. **Saliva is composed of biomolecules and fluids from different sources. Saliva is mainly secreted by salivary glands, and its informative biomolecules (DNA, RNA, proteins, metabolites and microbiota) are obtained from salivary glands, oral mucosa cells, oral microbiota and gingival crevicular fluid.**

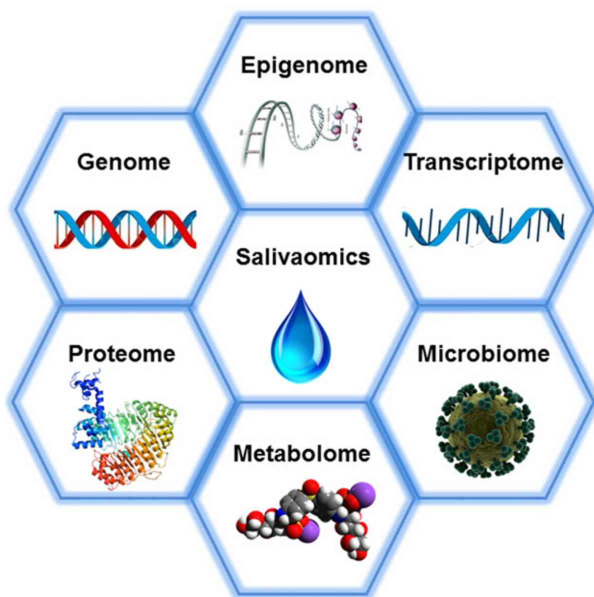

Fig. 2. **The components of salivaomics. Salivaomics as the various 'omics' constituents of saliva, including genomics (human and microbial), the oral microbiome, epigenome (DNA methylation), transcriptome (mRNA, microRNA and other noncoding RNAs), proteome and metabolome.**

saliva from healthy controls. Gene panels of four to 10 genes were constructed and exhibited a sensitivity of 62–77% and a specificity of 83–100% for oral squamous cell carcinoma. Carvalho et al. (18) evaluated tumor suppressor gene promoters in pretreatment saliva from patients with head and neck squamous cell carcinoma using quantitative methylation-specific PCR; methylation of at least one of the selected genes in the saliva DNA was demonstrated in more than 50% of patients, and local disease control and overall survival were significantly lower in patients presenting with hypermethylation in saliva rinses.

Transcriptomes (mRNA and microRNA)

Transcriptome studies have focused mainly on mRNA and microRNA, which are secreted from cells and enter the oral cavity from various sources, including salivary glands, gingival crevicular fluid and desquamated oral epithelial cells (74). The transcription of specific mRNA and microRNA is altered in disease states. Recent research has revealed more than 3,000 species of mRNA and over 300 microRNAs in the saliva of healthy and diseased subjects (6), suggesting that transcriptomic analysis can be of value to monitor healthy and disease states.

The human salivary transcriptome was first discovered in our laboratory using microarray technology, allowing high-throughput analysis (61). We then developed direct saliva transcriptome analysis to permit simple stabilization of salivary RNA and direct analysis without further processing (44, 56). Since 2010, we have reported the detection of salivary mRNA biomarkers in a number of cancers and systemic diseases. Using the Affymetrix HG-U133-Plus-2.0 array Affymetrix, Santa Clara, CA, USA) for discovery and quantitative real-time PCR for validation, Zhang et al. (113) identified four mRNA biomarkers (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, methyl-CpG binding domain protein 3-like 2, acrosomal vesicle protein 1 and dolichyl-phosphate mannosyltransferase polypeptide 1) that can differentiate patients with early-stage resectable pancreatic cancer from noncancer subjects (i.e. patients with chronic pancreatitis and healthy controls) with high sensitivity and specificity. Aside from pancreatic cancer, results from our laboratory further demonstrate the utility of salivary mRNAs for the

detection of oral cancer (60), lung cancer (115), breast cancer (114), ovarian cancer (57) and other systemic diseases. Hu et al. (43) reported that three mRNA biomarkers (myeloid cell nuclear differentiation antigen, guanylate binding protein 2 and low-affinity IIIb receptor for the Fc fragment of IgG) were significantly elevated in patients with primary Sjögren's syndrome compared with patients with systemic lupus erythematosus and healthy controls. Using the Illumina Human 12WG Expression BeadChip (Illumina, San Diego, CA, USA) for discovery and quantitative real-time PCR for validation, Lakschevitz et al. (51) demonstrated up-regulated expression of a variety of B-cell lymphoma-2-related transcripts in neutrophils from oral rinse samples of patients with chronic periodontitis compared with healthy controls. Gomes et al. (31) reported that, in saliva of patients with chronic periodontal disease and type 2 diabetes mellitus, the expression of mRNA for interferon gamma had an increasing trend association with severity of periodontitis.

MicroRNAs are a group of small, noncoding RNAs (19–25 nucleotides) that are encoded by genes but are not translated into proteins. MicroRNAs are centrally involved in various biological processes, including cell differentiation, proliferation and survival (5). Many studies have shown that microRNAs are frequently dysregulated in cancer tissues compared with healthy control tissues (49, 99, 116). Compared with salivary mRNA, salivary microRNAs are more stable (27, 76), and the fold change in microRNA between cancer cells and normal cells is fairly large (48, 72). Park et al. (76) used reverse transcription–preamplification–quantitative PCR to measure salivary microRNAs in patients with oral squamous cell carcinoma and in healthy controls. They found that two microRNAs (miR-125a and miR-200a) were significantly reduced in the saliva of patients with oral squamous cell carcinoma compared with healthy controls. Matse et al. (68) investigated differences of microRNA expression in saliva from patients with malignant and benign parotid gland tumors using the TaqMan microRNA array cards for discovery and quantitative real-time PCR for the validation phase. Their results indicated that a combination of four microRNAs (hsa-miR-132, hsa-miR-15b, mmu-miR-140 and hsa-miR-223) is valuable in the detection of parotid gland malignancy.

To advance the discovery of extracellular RNA biomarkers in saliva, massive parallel sequencing of transcripts (using RNA-Seq) was used to sequence and characterize the salivary transcriptome in greater detail (75). RNA-Seq is analytically more sensitive than microarrays, can detect differentially expressed genes and provides information about each RNA sequence that we were unable to obtain in previous studies (38, 104). Approximately 20–25% of the sequenced reads from cell-free saliva correspond with those for the human genome, and approximately 30% of the sequenced reads correspond with the Human Oral Microbiome Database. More than 4,000 coding and noncoding genes in cell-free saliva and whole saliva were detected (90).

The proteome

Saliva contains more than 2,000 proteins that are involved in many biological functions to maintain oral homeostasis (4). Unlike the relatively stable status in serum, proteins in saliva appear to be more susceptible to biochemical processes and degradation (35, 84). Esser et al. (25) reported that salivary protein degradation occurs rapidly, even during saliva collection and handling, which may compromise its clinical usefulness. Our laboratory has developed methods to stabilize the salivary proteome with protease inhibitors; as a result, we can keep salivary proteins stable for 2 weeks when stored at 4°C without significant degradation and without affecting downstream applications (108).

Because of high-sensitivity and high-accuracy mass measurement of peptides, mass spectrometry has become the core technology for protein identification. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry has proven to be a powerful tool for identification and quantification of post-translational modifications on proteins in saliva. Several studies have reported biomarkers in saliva using this rapid and high-throughput tool (20, 73, 93). Two-dimensional difference gel electrophoresis combined with mass spectrometry has recently been used in salivary proteomic biomarker discoveries. Using two-dimensional difference gel electrophoresis, Hu et al. (40, 41) reported 16 peptides in saliva that were found at significantly different levels in patients with primary Sjögren's syndrome compared with healthy controls. Xiao et al. (109) reported three proteins (zinc-α-2-glycoprotein, haptoglobin and human calprotectin) that had good discriminatory power in lung cancer patients and healthy control subjects, with high sensitivity (89%) and high specificity (92%). Studies also detected salivary protein biomarkers, such as S100 calcium binding protein P, plastin-2 and neutrophil defensin, in chronic periodontitis (83); interleukin-8, interleukin-1beta and Mac-2 binding protein in oral cancer (24); and adenosine deaminase in tongue cancer (79).

The metabolome

The metabolome, which enables the parallel evaluation of a group of endogenous and exogenous metabolites, including lipids, amino acids, peptides, nucleic acids, organic acids, vitamins, thiols and carbohydrates, is a valuable tool for discovering biomarkers, monitoring physiological status and making proper treatment decisions (3, 95, 112).

Based on the different metabolomic technology, studies have reported that salivary metabolites cannot only identify health status (7, 8), but can also discriminate diseased patients from healthy control subjects. Sugimoto et al. (94) used capillary electrophoresis time-of-flight mass spectrometry to investigate discriminatory metabolites from healthy controls and patients with oral cancer, pancreatic cancer, breast cancer and periodontal disease, and identified 57 metabolites predictive of each individual disease. Most of these metabolites were present at relatively high concentrations in all three cancer-patient groups compared with the periodontal disease patients and healthy control subjects. In addition, three metabolites (taurine, piperidine and a peak at 120.0801 m/z) were oral cancer-specific markers with an area under the curve of 0.865, and eight metabolites (leucine, isoleucine, tryptophan, valine, glutamic acid, phenylalanine, glutamine and aspartic acid) were pancreatic cancer-specific markers with an area under the curve of 0.993. Also using capillary electrophoresis time-of-flight mass spectrometry, Tsuruoka et al. (98) demonstrated that the concentrations of two salivary metabolites (arginine and tyrosine) differed significantly between patients with dementia and healthy subjects. Applying ultraperformance liquid chromatography coupled with quadrupole/time-of-flight mass spectrometry, Wei et al. (105) found that a combination of three salivary metabolites (phenylalanine, valine and lactic acid) could distinguish patients with oral squamous cell carcinoma from healthy controls with high sensitivity and high specificity (86.5% and 82.4%, respectively), and could also distinguish patients with oral leukoplakia from healthy controls with high sensitivity and high specificity (94.6% and 84.4%, respectively).

The microbiome

The oral cavity is a diverse habitat of bacteria and other microorganisms. A series of evidence shows that oral dysbiosis can lead to oral diseases, such as periodontal diseases (29) and dental caries (15), as well as cancer and other systemic diseases (1, 21, 85). In the past, it was thought that there were approximately 1,000 bacterial species in the oral cavity (82). Now, using next-generation sequencing technology, the number of species could reach more than 10,000 (62). The use of next-generation sequencing, bacterial microarrays and other emerging techniques can advance the investigation of the salivary microbiome and identify the association between special bacteria or other microorganisms and special oral or systemic diseases (39, 110).

Mager et al. (65) used checkerboard DNA–DNA hybridization to evaluate the oral microbiota in saliva from patients with oral squamous cell carcinoma and healthy subjects, and found a combination of three microbiotas (*Capnocytophaga gingivalis, Prevotella melaninogenica* and *Streptococcus mitis*) that could be used as diagnostic biomarkers with 80% sensitivity and 82% specificity. Recently, using the Human Oral Microbe Identification Microarray during the discovery phase and quantitative PCR during the validation phase, Farrell et al. (26) profiled the salivary microbiota from patients with pancreatic cancer and healthy subjects; the results showed that the numbers of 31 bacterial species were increased and the numbers of 25 were decreased in patients with pancreatic cancer compared with healthy subjects and that two bacterial candidates (*Neisseria elongate* and *Streptococcus mitis*) could be used to distinguish patients with pancreatic cancer from healthy controls with 96.4% sensitivity and 82.1% specificity.

The mechanism of salivary diagnostics

Recent translational salivary biomarker development studies have supported that salivary biomarkers can discriminate patients with oral and systemic diseases from subjects without such diseases (12, 13, 57, 113, 115). However, the mechanisms of how diseases distal from the oral cavity lead to the appearance of discriminatory biomarkers in saliva are largely unclear (54). Investigating the origin of salivary biomarkers will be a significant goal in the development of salivary diagnostics, and the mechanisms of salivary diagnostics need to be elucidated.

Studies have increasingly demonstrated that some salivary biomarkers might derive from systemic sources. Gao et al. (28) used mouse models of cancer to determine whether salivary biomarker profiles are affected by distal tumor development. Their data analysis of nerve growth factor production and the

transcription factor (early growth response-1) suggests that the production of growth factors in tumor tissue represents one mechanism by which a distant tumor can alter the transcriptome of the salivary glands, and hence saliva. Although their report did not comprehensively demonstrate the mechanistic connection between systemic disease development and salivary biomarker alterations, it did begin to paint a picture of the concept of the existence of systemic networks in the human body that allow communication between distal diseases and the salivary glands. Signals transmitted through such networks might induce related signaling pathways that result in altered gene expression and protein translation, and thereby produce disease-induced salivary biomarker profiles. Therefore, the salivary transcriptomic profile might be composed of transcripts that originate in distant diseased tissues as well as of transcripts that originate in salivary glands, and transcription factors that originate in distant tissues might alter the expression levels of these transcripts.

Exosomes: from formation to target

Cells continuously secrete a large number of microvesicles, macromolecular complexes and small molecules into the extracellular space. Among the microvesicles secreted, nanoparticles called exosomes are currently undergoing intense scrutiny. Although exosomes were first discovered nearly 30 years ago, they were considered as little more than cellular entities that acted to discard unwanted molecular components. However, since 2007, evidence has begun to accumulate to suggest that these vesicles are signaling shuttles containing cell-specific collections of proteins, lipids and genetic material that are transported to other cells, in which they alter function and physiology. Hence, these findings have reignited interest in exosomes (97).

Exosomes are small vesicles (30–120 nm) that contain nucleic acid and protein, and they are perceived to carry this cargo between diverse locations in the body. They are distinguished in their genesis by being budded into endosomes to form multivesicular bodies in the cytoplasm. Exosomes are released to extracellular fluids by fusion of these multivesicular bodies with the cell surface, resulting in secretion in bursts. They are secreted by most cell types (23, 100, 117), and are also found in abundance in body fluids, including blood, saliva, urine and breast milk (103).

Specifically, at the beginning of exosome formation, internal vesicles are formed by the inward budding of cellular compartments known as multivesicular endosomes. Multivesicular endosomes bud inward to form small internal vesicles that contain proteins, mRNA and microRNA from the cytoplasm. When multivesicular endosomes fuse with the cell membrane, these internal vesicles are released as exosomes, which can travel to distant tissues to influence various aspects of cellular behavior; alternatively, multivesicular endosomes can fuse with lysosomes, which degrade multivesicular endosome contents. Upon reaching their destinations (which are usually determined by the binding of specific ligands on their surfaces), exosomes enter the target cells in one of two ways: by being taken up by the target cell's endocytic pathway; or by fusing to the target cell's membrane and releasing their contents directly into the cytoplasm. Cells also secrete other membrane-derived vesicles, such as ectosomes, shed vesicles or microvesicles, which bud directly from the cell's plasma membrane. These vesicles are also known to carry active proteins and RNAs, as well as some compounds that have never before been described in exosomes; however, little is known about their effects on distant tissues (Fig. 3) (97).

Biological functions of exosomes

Exosomes have been proposed to signal both by binding to cell-surface receptors through adhesion molecules (80) and by fusing with, or being internalized by, the recipient cell, potentially donating their own cytoplasm to the recipient cell (77, 96). The latter implies that exosomes may have mechanisms that are different from their function in the immune system. Lässer et al. (52) recently discovered substantial amounts of RNA in exosomes derived from mast cells, which have the capacity to donate their RNA to other cells and can subsequently affect the protein production of a recipient cell. This finding suggests that RNA can be transferred between mammalian cells by an extracellular exosome-based transport mechanism, which has vast implications for the understanding of cell communication, regulation and signaling, in addition to extensive therapeutic potential in many diseases.

Beyond their characteristic repertoire of surface markers, exosomes feature a wide range of surface and internal proteins specific to their source. When considering the diversity of cargo transported by exosomes, it should come as no surprise that exosomes have already been implicated in the development of polarized epithelial cells, neuronal development and

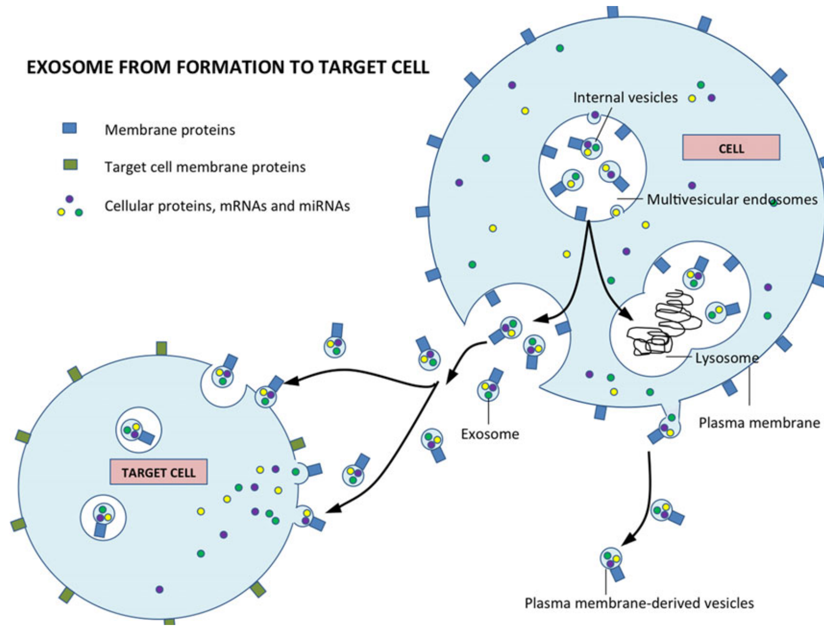

Fig. 3. **Exosomes: from formation to target cell. Multivesicular endosomes bud inward to form small internal vesicles that contain proteins, mRNAs and microRNAs from the cytoplasm. Multivesicular endosomes can fuse with the cell membrane and internal vesicles are released as exosomes. Alternatively, multivesicular endosomes can fuse with lysosomes, which degrade the contents of the multivesicular endosome. Exosomes can travel to distant tissues and enter target cells by being taken up by the target cell's endocytic pathway or by fusing to the target cell's membrane and releasing their contents directly into the target cell's cytoplasm.**

tumor growth (50). Hunter et al. (45) identified the presence of various microRNAs in human serum exosomes. A single microRNA can regulate hundreds of genes and may act as a master regulator of processes; therefore, select subsets of microRNAs can be used as biomarkers of physiological and pathological states. Another feature that makes microRNAs excellent candidates for use in biomarker studies is their remarkable stability and resistance to degradation. In the clinical setting, exosomes are present in blood, saliva, plasma, urine, amniotic fluid and effusions from malignant tumors. Given the relative ease and noninvasive nature of exosome isolation from patient samples, as well as their distinctive protein and nucleotide contents, several studies have suggested using exosomal biomarkers for disease diagnostic purposes. A study by Mi et al. (70) showed that the expression of as few as two microRNAs can accurately discriminate acute lymphoid leukemia from acute myeloid leukemia, whilst Skog et al. (88) suggested that glioblastoma tumor-derived exosomes in patient with glioblastoma tumor carry a distinctive microRNA payload that can be used diagnostically. The majority of these studies investigated exosomes isolated from serum; only a few have focused on proteomic exosomal biomarkers in urine and saliva (71).

Diagnostic and therapeutic potential of exosomes

Recent research indicates that exosomes provide a mechanism for identifying diagnostic and therapeutic salivary biomarkers that perform well in pancreatic cancer, breast cancer and glioblastoma. Lau et al. (53) examined the hypothesis that pancreatic tumor-derived exosomes are mechanistically involved in the development of pancreatic cancer-discriminatory salivary transcriptomic biomarkers. They developed a pancreatic cancer mouse model that yielded discriminatory salivary biomarkers by implanting the mouse pancreatic cancer cell line, Panc02, into the pancreas of the syngeneic C57BL/6 host. Then, they tested the role of pancreatic cancer-derived exosomes in the development of discriminatory salivary biomarkers by engineering a Panc02 cell line that is suppressed for exosome biogenesis, implanting it into the C56BL/6 mouse and examining whether the discriminatory salivary biomarker profile was ablated or disrupted. Suppression of exosome biogenesis resulted in the ablation of discriminatory salivary biomarker development. Their study supported the hypothesis that tumor-derived exosomes provide a mechanism for the development of discriminatory salivary biomarkers that are applicable to distal systemic diseases (53).

Lau et al. (54) used an *in vitro* breast cancer model to demonstrate that breast cancer-derived exosome-like microvesicles are capable of interacting with salivary gland cells, altering the composition of their secreted exosome-like microvesicles. They found that the salivary gland cells secrete exosome-like microvesicles which encapsulate both protein and mRNA. They also showed that breast cancer-derived exosome-like microvesicles communicate with and activate the transcriptional machinery of salivary

gland cells. Thus, the interaction altered the composition of the salivary gland cell-derived exosome-like microvesicles at both transcriptome and proteome levels.

Translational and clinical applications of saliva biomarkers

As we have increased our understanding of salivaomics and the advantages of saliva as a valuable diagnostic tool have been revealed, the surveillance of disease and general health has become a highly desirable goal. Evaluating alterations of salivary biomarkers can be applied to the early detection, risk assessment, diagnosis, prognosis and monitoring of the progress of a variety of diseases, including cancers, infectious diseases and immune diseases. Some oncogenic proteins that are detectable in saliva (e.g. human epidermal growth factor receptor-2) provide a basis for developing targeted therapy. Saliva also serves as a platform for personalized medicine. By comparing salivary biomarkers from patients receiving different treatments with different outcomes, saliva proteomics can also be used to monitor treatment response.

Disease detection

Oral disease and oral cancer

Salivary diagnostics have been developed to monitor periodontal disease and other oral diseases. Salivary interleukin-1beta and multiple oral pathogens were found to be associated with periodontitis in a study in which the concentrations of a select subset of salivary proteins (elastase, lactate dehydrogenase, interleukin-1beta, interleukin-6 and tumor necrosis factor alpha) and the presence of five pathogens were compared in patients with advanced periodontal disease and healthy controls (32) (Table 1). In a study on dysplastic oral leukoplakia in relation to tobacco habits and periodontitis, increasing salivary interleukin-6 levels were demonstrated to correlate with severity of dysplasia (87). These findings indicate that salivary biomarkers have the potential to detect oral disease and determine disease stage.

Oral cancer (more than 90% of which are oral squamous cell carcinoma) is the sixth most common cancer worldwide, with an average 5-year survival rate of approximately 60% (111). The key to reduce the mortality and morbidity associated with this disease is to develop strategies to identify oral squamous cell carcinoma at an early stage. Several biomarker candidates for oral squamous cell carcinoma have been reported, including endothelin receptor type B hypermethylation (78), interleukin-8 and microRNAs (such as miR-200a, miR-125a and miR-31) (63, 76, 91); however, only interleukin-8 has proved to be a cost-efficient analyte for early detection of oral squamous cell carcinoma (9). Several biomarker panels have also been investigated for their ability to detect oral squamous cell carcinoma. Our previous salivary transcriptomic studies have discovered seven oral squamous cell carcinoma-associated salivary RNAs (interleukin-8, interleukin-1beta, dual specificity phosphatase 1, H3 histone family 3A, ornithine decarboxylase antizyme 1, S100 calcium binding protein P and spermine N1-acetyltransferase). Initially, the levels of these RNAs were measured in a training set of 32 oral squamous cell carcinomas and 32 control samples using quantitative PCR, and a logistic regression model including four markers (interleukin-8, Spermidine/spermine N1-acetyltransferase, interleukin-1beta and ornithine decarboxylase antizyme 1) achieved a cross-validation prediction accuracy rate of 81%, showing their potential as biomarkers for oral squamous cell carcinoma detection (60). The prevalidation study of these biomarkers demonstrated their ability to discriminate patients with oral squamous cell carcinoma from control subjects (24). Other proteomic, metabolomic or epigenetic biomarkers have achieved lower specificity or have not been validated (11, 42, 94, 102, 105).

Pancreatic cancer

Pancreatic cancer is characterized by a propensity to disseminate rapidly to the lymphatic system and distant organs. Approximately 15–20% of patients have surgically resectable disease at the time of presentation; however, of these patients, only approximately 20% survive to 5 years (36, 47, 58). This aggressive biology, resistance to conventional and targeted therapeutic agents and lack of biomarkers for early detection result in the poor outcomes of these patients. In a significant milestone, prospective sample collection and a retrospective double-blind validation study (113) showed that a salivary transcriptome profile could be used to detect early-stage resectable pancreatic cancer. Microbial profiling derived from the Human Oral Microbe Identification Microarray was used to investigate variations in salivary microbiota between groups of 10 resectable patients with pancreatic cancer and 10 matched healthy controls. The

Table 1. Salivary biomarkers for detecting oral disease, oral cancer, pancreas cancer, lung cancer, and breast cancer, discovered using epigenomics, transcriptomics, proteomics and metabolomics

Disease	Methodology	Approach	Markers
Periodontal disease	Proteomic	ELISA (87)	Interleukin-6
	Proteomic and microbial studies	ELISA (32)	Interleukin-1 and *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia* and *Treponema denticola*
	Proteomic	Multiplex protein array discovery (55)	Interleukin-6, interleukin-8
Oral cancer	Epigenomics	Candidate from previous study, quantitative methylation-specific PCR discovery and validation (78)	Kinesin family member 1A, endothelin receptor type B
	Transcriptomics	Microarray discovery and quantitative PCR validation (60)	Interleukin-8, spermine N1-acetyltransferase, interleukin-1beta, ornithine decarboxylase antizyme 1, H3 histone family 3A, dual specificity phosphatase 1, S100 calcium binding protein
	Transcriptomics	Microarray discovery and quantitative PCR validation (24)	Interleukin-8, interleukin-1beta, ornithine decarboxylase antizyme 1, spermine N1-acetyltransferase
	Transcriptomics	Discovery and validation by reverse transcription–preamplification–quantitative PCR or candidate gene selection based on previous study, quantitative RT-PCR quantification (63, 76)	miR-200a, miR-125a and miR-31
	Proteomic	ELISA assessment and quantitative PCR confirmation (91)	Interleukin-8
Pancreatic cancer	Transcriptomics	Affymetrix array discovery and quantitative RT-PCR validation (113)	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, methyl-CpG binding domain protein 3-like 2, acrosomal vesicle protein 1, dolichyl-phosphate mannosyltransferase polypeptide 1
	Metabolomics	Discovery by capillary electrophoresis time-of-flight mass spectrometry-based metabolomics (94)	Leucine with isoleucine, tryptophan, valine, glutamic acid, phenylalanine, glutamine and aspartic acid
	Microbiome	Microbial profiling using the Human Oral Microbe Identification Microarray (26)	*Neisseria elongata* and *Streptococcus mitis*
Lung cancer	Transcriptomics	Microarray discovery and quantitative RT-PCR verification and prevalidation (115)	Cyclin I, epidermal growth factor receptor, fibroblast growth factor 19, fibroblast growth factor receptor substrate 2, growth regulation by estrogen in breast cancer 1
	Proteomics	Two-dimensional gel electrophoresis and liquid chromatography–tandem mass spectrometry (109)	Calprotectin, zinc-α-2-glycoprotein, haptoglobin
	Metabolomics	Discovery by surface-enhanced Raman spectroscopy (59)	Unidentified peak wavelengths: 822, 884, 909, 925, 1009, 1,077, 1,369, 1,393 and 1,721 per cm
Breast cancer	Combination proteomic/ transcriptomic approaches	Discovery by two-dimensional difference gel electrophoresis and RT-PCR/Affymetrix; validation by quantitative RT-PCR (114)	mRNAs: cystatin A, tumor protein, translationally-controlled 1, insulin-like growth factor 2 mRNA binding protein 1, glutamate receptor metabotropic 1, glutamate receptor ionotropic kainate 1, hexose-6-phosphate dehydrogenase, Mdm4 p53 binding protein S100A8 Protein: carbonic anhydrase 6

profiling of bacterial candidates was further validated by performing quantitative PCR on samples from an independent cohort of 28 patients with resectable pancreatic cancer, 28 matched healthy controls and 27 patients with chronic pancreatitis, which yielded a receiver–operating characteristics plot area under the curve value of 0.90 (95% CI: 0.78–0.96; $P < 0.0001$) (26). A metabolomic approach also identified pancreatic cancer-specific salivary metabolomic biomarkers that can distinguish pancreatic cancer from oral cancer, breast cancer and cancer-free controls (94). Both microbiomic and metabolomic biomarkers have achieved excellent accuracy for pancreatic cancer, although only the microbiomic panel has been validated.

Lung cancer

Lung cancer is the most common cause of cancer-related death in men and women, in part because symptoms are frequently absent until the disease has already metastasized. Early detection represents a very promising approach to reducing the incidence and mortality of lung cancer. However, conventional diagnostic methods for lung cancer are unsuitable for widespread screening because they are expensive and occasionally miss tumors or invasive cancer (30, 37). Although computed tomography has been widely used for screening for early lung cancer, it is associated with a high false-positive rate (101). Biomarkers for lung cancer have the potential to improve early detection beyond the use of computed tomography scans (34). Using two-dimensional difference gel electrophoresis proteomic analysis of saliva samples from patients with lung cancer, 16 candidate biomarkers have been discovered and further verified (109). Three candidate markers (calprotectin, zinc-α-2-glycoprotein and haptoglobin) achieved good sensitivity and excellent specificity and accuracy. Moreover, a transcriptomic biomarker profile, including the B-Raf gene (which is involved in directing cell growth), cyclin I (which binds activated cyclin-dependent kinase 5), the epidermal growth factor receptor, fibroblast growth factor 19, fibroblast growth factor receptor substrate 2, growth regulation by estrogen in breast cancer 1 and leucine zipper putative tumor suppressor 1, has been identified, and a panel consisting of five of these markers is able to differentiate lung cancer patients from cancer-free subjects with 93.75% sensitivity and 82.81% specificity (115). Surface-enhanced Raman spectroscopy was recently applied to identify biomarkers for lung cancer and revealed nine peaks (assigned to amino acids and nucleic acid bases) that are able to distinguish samples from patients with lung cancer and cancer-free controls with 86% accuracy, 94% sensitivity and 81% specificity (59). Therefore, both transcriptomic and proteomic approaches are proving highly useful in developing biomarkers for lung cancer.

Breast cancer

Breast cancer is the most common form of cancer and the second leading cause of cancer deaths in women in the USA (46). Today, breast cancer detection depends on physical examination and imaging studies. Earlier investigations demonstrated the potential for salivary proteomic detection of breast cancer (e.g. using the salivary protein c-erbB-2) (92, 93). Recently, isobaric tags for relative and absolute quantitation technology, combined with liquid chromatography-tandem mass spectrometry, was used to analyze saliva samples collected from 20 breast cancer patients and 10 healthy controls. Nine proteins were associated with breast cancer and exhibited 1.5-fold up-regulation or down-regulation (16). The Affymetrix HG-U133-Plus-2.0 Array and two-dimensional difference gel electrophoresis were used to analyze the salivary transcriptomes and proteomes of 10 breast cancer patients and 10 matched controls during the discovery phase and the salivary transcriptomes and proteomes of 30 breast cancer patients and 63 controls during prevalidation (114). Eight mRNA biomarkers and one protein biomarker were identified, yielding an accuracy of 92% (83% sensitivity and 97% specificity) for the preclinical validation sample set.

Other systemic diseases

In addition to providing powerful biomarkers to detect systemic cancers, saliva also provides biomarkers for autoimmune diseases, systemic microbial infections and diabetes (9). Although present at significantly lower levels than in serum, hepatitis C virus RNA can be consistently detected in saliva from hepatitis C virus-infected individuals using quantitative PCR (69). Label-free differential protein expression analysis using multidimensional liquid chromatography–tandem mass spectrometry was conducted to characterize the proteome of saliva collected from patients with type 2 diabetes compared with nondiabetic controls. Several proteins were found to have diagnostic potential for type 2 diabetes, but these require additional study (10).

Personalized medicine

Cancer patients can be classified according to altered protein expression profiles, and statistical methodologies can be used subsequently to develop predictors for subgroups of patients who may benefit from targeted therapy. Trastuzumab is a humanized monoclonal antibody targeted against HER2, which is over-expressed in 25–30% of patients with breast cancer. HER2 over-expression is an indicator for trastuzumab therapy. United States Food and Drug Administration-approved immunohistochemical and fluorescence *insitu* hybridization methods (106) are now available to assess HER2 over-expression; however, these methods are only semiquantitative and are interpreted in an operator-dependent manner. Because HER2 is detectable in human saliva, incorporation of this marker in clinical trials might assist in the classification of breast cancer patients and help determine which patient subgroups are most likely to benefit from such a molecular-targeted therapy. Saliva is also applicable for noninvasive prenatal diagnosis in predictive, preventive and personalized medicine, especially for infants born prematurely. Amplification of salivary RNA and microarray analysis identified 9,286 gene transcripts that exhibited statistically significant changes in expression across individuals over time (66). The changes in gene expression were closely linked to developmental pathways. According to Ingenuity Pathway Analysis, a total of 2,186 genes are involved in successful oral feeding of infants (67). This finding elucidates the biological processes involved in oral feeding in the newborn at a molecular level, as well as novel pathways associated with successful oral feeding. A gingivitis-focused experiment that employed a multiplex protein array for selected biomarkers implicated in host defense, inflammation, tissue destruction and angiogenesis demonstrated that salivary biomarkers can also be used to evaluate the host response to bacterial invasion. Salivary interleukin-6 and interleukin-8 levels were shown to provide the best distinction between high and low responders (55).

Therapeutic efficiency

Several candidate approaches have elucidated biomarkers for periodontitis and responses to therapy. In a study aiming to identify salivary biomarkers for chronic periodontitis, 33 participants received oral hygiene instructions alone and 35 received oral hygiene instructions in combination with conventional periodontal treatment comprising scaling and root planing. The levels of interleukin-1beta, macrophage inflammatory protein-1alpha, matrix metalloproteinase-8 and osteoprotegerin detected in saliva reflected disease severity and response to therapy (86).

Conclusion

To be useful in a clinical setting, biomarkers should accurately reflect the presence of disease in a reproducible manner and be able to be collected using a noninvasive method that requires little preparation. Salivary diagnostics have these characteristics, and abundance of biomarkers in saliva are applicable for detecting systemic disorders in addition to oral diseases. The current decision to use available diagnostic methods for many diseases is based on patients' symptoms and clinical information. The process of obtaining a final diagnosis can impose a burden on hospitals and involve a long waiting time for patients. As an accessible and noninvasive primary test for diseases, salivary diagnostics reduces the hospital's burden and the use of unnecessarily invasive procedures. As methods of stabilizing whole saliva are developed, salivary diagnostics can be performed correctly in the clinic without specially trained professionals, using point-of-care technology to allow the detection of disease without any preparation.

As detailed above, salivary diagnostics have both translational and clinical potentials. The development of high-throughput technology has revealed advanced insights toward an understanding of saliva as a reflection of the condition of the whole body. Exosomes provide a mechanism for the expression of diagnostic biomarkers in saliva, and also promote saliva as a powerful and unprecedented diagnostic tool, in combination with high-throughput technology and bioinformatics platforms. The interpretation and utilization of this information will bolster the applicability of saliva in diagnosing disease, evaluating therapies and designing personalized medicine.

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Conflict of interests

David Wong is co-founder of RNAmeTRIX Inc., a molecular diagnostics company. He holds equity in RNAmeTRIX, and serves as a company director and scientific advisor. The University of California also holds equity in RNAmeTRIX. Intellectual property that David Wong invented, and which was patented by the University of California, has been licensed to RNAmeTRIX. Additionally, he is a consultant for PeriRx, GlaxoSmithKline and Wrigley. No other authors declare competing interests.

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