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Saliva Diagnostics

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Abstract

Cancer remains one of the leading causes of death, and early detection of this disease is crucial for increasing survival rates. Although cancer can be diagnosed following tissue biopsy, the biopsy procedure is invasive; liquid biopsy provides an alternative that is more comfortable for the patient. While blood, urine, and cerebral spinal fluid can all be used as a source of liquid biopsy, saliva is an ideal source of body fluid that is readily available and easily collected in the most noninvasive manner. Characterization of salivary constituents in the disease setting provides critical data for understanding pathophysiology and the evaluation of diagnostic potential. The aim of saliva diagnostics is therefore to develop a rapid and noninvasive detection of oral and systemic diseases that could be used together with compact analysis systems in the clinic to facilitate point-of-care diagnostics.

Keywords

saliva liquid biopsy; salivaomics; saliva exosomics; salivary exosomes; circulating biomarkers

1. INTRODUCTION

Saliva is a critical bodily fluid required for the digestion of food and maintenance of good oral health. It contains secreted enzymes, hormones, cytokines, and antibodies that act as mediators of salivary functions. In addition, saliva contains microorganisms and cellular debris (1). There are three pairs of major salivary glands (parotid, submandibular, and sublingual) and many minor salivary glands dispersed throughout the oral mucosa (2). Owing to their proximity to blood vessels, the salivary glands are a rich source of metabolite exchange between the oral cavity and the circulatory system (3). Indeed, many proteins found in human serum can also be detected in saliva; this suggests that saliva could be used as a proxy for the measurement of disease-related circulating biomarkers (4). In the

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DISCLOSURE STATEMENT

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following review we provide a comprehensive overview of salivary biomarkers and the salivary exosomes in which they are encapsulated. We also describe a novel electrochemical sensing technology (electric field–induced release and measurement, or EFIRM) that can be used for biomarker detection and disease monitoring; this technology is pioneered to implement future point-of-care saliva testing methodologies.

2. SALIVAOMICS

Rising to prominence over the last decade, salivaomics refers to the integrated analysis of multiple large-scale molecular readouts from this important biological fluid (5, 6). Such readouts include genomics, epigenomics, transcriptomics, proteomics, microbiomics, and metabolomics. There are several challenges associated with obtaining a clear and disease-relevant signal from salivary samples. For example, components of food and the presence of bacteria in the oral cavity can contribute to noise in salivary data sets (7). The composition of saliva is also affected by natural circadian rhythms (8), the physical action of mastication (9), and the activity of amylase, which is abundant in saliva. RNAs (and many proteins, including histatins, statherin, and acidic prolinerich polypeptides) are also highly labile when taken out of the buffered environment of the saliva (10). Thus, methods to stabilize these molecular markers are paramount in order to preserve a representative snapshot of the true physiological state; thus, many protocols include protease and RNase inhibitors in extraction buffers. Finally, it is increasingly clear that analysis of bulk saliva samples may mask physiologically relevant signals from small subpopulations of cells or metabolites. Therefore, single-cell technologies or ultrasensitive methods of detection will ultimately be required to obtain more accurate information from saliva.

2.1. Salivary Genomics

Analysis of direct tumor biopsy is perhaps the most accurate approach for molecular diagnostics in cancer. However, taking tumor samples can be extremely uncomfortable for the patient, and repeat biopsy for disease monitoring may not always be possible. Furthermore, some deep-seated tumors may not be amenable to this approach. These disadvantages prompted the search for less-invasive methods for cancer diagnosis and surveillance. Circulating tumor DNA (ctDNA) is found in the serum and is composed of genomic DNA that is shed from the original tumor (11, 12). The cancer-specific mutational signatures of ctDNA can be differentiated from those of DNA from noncancerous tissues. Multiple studies have shown a high concordance between mutational profiles in ctDNA and those in the primary tumor (13-17). Furthermore, the relative abundance of ctDNA, or of specific mutations therein, can be used to monitor disease response to therapeutic intervention, and ctDNA samples can be taken repeatedly, which facilitates real-time surveillance of treatment (18-21). This ctDNA-based liquid biopsy approach is rapidly being adopted in many preclinical and clinical settings.

Although most liquid biopsies are taken by needle aspiration from the blood, it is possible that salivary fluid may offer an even less-invasive means of disease monitoring. The stability and relatively high quality of salivary DNA makes this an even more attractive possibility (22-24). Although diagnostics and monitoring using salivary ctDNA are in their infancy, a

recent study indicated their utility in head and neck squamous cell carcinoma (HNSCC) (25, 26). Key cancer-associated somatic mutations and the presence of human papillomaviruses (HPV16 and 18) were evaluated among a cohort of 93 HNSCC patients (including 20 patients with early disease; see Table 1 for details). While plasma ctDNA analysis was associated with higher sensitivity for oropharynx, hypopharynx, and larynx cancers (plasma ctDNA: 86–100% versus salivary ctDNA: 47–70%), salivary ctDNA analysis had greater sensitivity for the detection of oral cancer (100% versus 80%). The latter result is likely attributable to the close physical proximity of salivary fluid to the actual tumor. Combined analysis of plasma and salivary ctDNA yielded a 96% detection rate, irrespective of tumor location or stage. Together, these data indicate that the optimal combination of bodily fluids used for ctDNA analysis should be chosen on a tumor type–specific basis.

2.2. Salivary Transcriptomics

Saliva contains diverse types of RNA transcripts, including messenger RNA (mRNA), piwi-interacting RNA (piRNA), and micro RNA (miRNA) (27, 28). Although the value of piRNA as a biomarker remains to be determined, both mRNA and miRNA within the saliva have been used to detect several cancers, including pancreatic (29), breast (30), ovarian (31), and lung malignancies (32). We highlight some examples below and in Table 2.

Profiling of saliva samples from patients with oral squamous cell carcinoma identified multiple mRNA biomarkers (33). A subset of four mRNAs (*IL1B*, *OAZ1*, *SAT*, and *IL8*) was sufficient for use in a logistic regression model to provide 91% sensitivity and 91% specificity for the detection of cancer.

Salivary miRNAs are packaged in salivary exosomes, where they are protected from RNase-dependent degradation (34, 35). Consistent with the general dysregulation of miRNAs in tumor cells themselves, the levels of specific miRNAs in the saliva of cancer patients are altered in comparison to those of healthy individuals. For example, the levels of miR-125a and miR-200a were significantly lower in saliva from oral cancer patients than healthy patients (36). Conversely, the levels of miR-27b and miR-31 were significantly higher in the saliva of oral cancer patients (37, 38). miR-139 and miR-31 reverted to baseline levels following excision of the malignant lesions, suggesting that these miRNAs could serve as prognostic biomarkers (37, 39).

Despite these intriguing findings, further preclinical and clinical studies are required to validate the roles of salivary miRNAs as disease biomarkers. It will be especially important to standardize the way salivary exosome miRNAs are detected and analyzed. Furthermore, researchers must find ways to deconvolute salivary miRNA signals that originated in immune cells versus tumor or salivary cells. This is critical, because systemic or local inflammation may perturb miRNA expression and generate variability, even within the same individual (40). It will be useful to crossreference data from future studies with the miRNA database, miRandola, which is a large catalog of extracellular noncoding RNAs found in a variety of diseases (http://mirandola.iit.cnr.it/) (41).

2.3. Salivary Proteomics

To the best of our knowledge, the first attempt at cancer diagnosis using salivary protein was made by Hoerman et al. (42) more than 60 years ago; the group showed that prostate cancer patients had elevated acid phosphatase enzymatic activity in parotid saliva. Since then, the advent of high-throughput mass spectrometry combined with bioinformatics has given rise to the field of proteomics, which holds great promise for disease detection and monitoring.

Although in its infancy, there are clear signs that salivary proteomics will prove extremely useful. For example, a US-based consortium has generated a comprehensive catalog of the salivary proteome of healthy individuals, identifying 1,166 proteins in parotid and submandibular/sublingual gland ductal saliva (43). The data are publicly available via the Human Salivary Proteome Wiki (https://salivaryproteome.nidcr.nih.gov). Between 20% and 30% of the salivary proteome overlaps with the plasma proteome, indicating that many salivary constituents are derived from the blood (4, 44). This observation, together with the close physical proximity of saliva and blood, suggests that saliva could be used as a proxy to detect disease. Unlike serum proteins, salivary proteins appear to be more susceptible to degradation (10, 45). Indeed, they degrade rapidly even during saliva collection and handling, which may compromise downstream experiments and limit the application of saliva-based methods (46). Protease inhibitors can be used to stabilize salivary proteins, thereby enabling the storage of saliva samples for up to two weeks without significant degradation (47). Table 3 summarizes salivary proteins that may have potential utility as biomarkers for cancer detection or disease monitoring.

3. SALIVA EXOSOMICS: NEXT-GENERATION SALIVAOMICS

Exosomes are nanosized extracellular vesicles with a diameter between 30 and 100 nm that have been isolated from virtually all types of body fluid, including saliva (48, 49). They are derived from endosomal membranes and are shuttled to the extracellular space during exocytosis. They are critical transporters of cell type–specific cargos that are delivered locally to the microenvironment and systemically via the vasculature. By relaying molecular information from their parental cell of origin to recipient cells, they play important roles in intercellular signaling and cellular homeostasis. Given their biological role in cancer pathogenesis, exosomes may harbor biomarkers that can be harnessed for detecting and monitoring cancer (50).

While exosomes are present in the saliva of healthy individuals, they may contain disease-related biomarkers from tumor cells that have been packaged and transported to the salivary glands (51). The use of these small but information-rich nanovesicles reduces the overall complexity of saliva (52). The term saliva exosomics defines the study of the genomic, transcriptomic, and proteomic features of exosomes and how they impact biological functions in oral and systemic diseases (7). Saliva exosomics is therefore considered next-generation salivaomics. Although the field is still in its infancy, it will undoubtedly reveal many novel facets of saliva biology as research gathers pace.

3.1. Salivary Exosomes

Salivary exosomes are nanoscale extracellular vesicles secreted by the salivary glands and oral epithelial cells (35, 53). Surrounded by a phospholipid bilayer, they carry many cell type–specific cargos (Figure 1). Prominent examples include tetraspanins, calcium-binding proteins, heat shock proteins, water channels, major histocompatibility complexes, and proteins associated with membrane fusion/trafficking (e.g., annexin, Rab GTPases) (54, 55). Almost half of salivary proteins are extracellular (e.g., immunoglobulin chains) or secretory (e.g., serum albumin), suggesting that they are derived from vesicles that originate from circulating lymphocytes and intravascular fluid (4, 44, 55).

Intriguingly, salivary exosomes play a role in the initiation of blood clotting (56). This is because they contain tissue factor, which works in concert with factor VII in the plasma to elicit coagulation. Salivary exosomes accelerate clotting in exosome-depleted plasma, and this can be attenuated by addition of anti-factor VII. These data highlight the importance of these exosomes in a critical physiological process.

Multiple types of RNA are found in salivary exosomes, where they are protected from RNase-dependent degradation (57). The exosomes therefore serve as an enriched source of RNA signaling mediators, primarily composed of piRNA (7.48%), miRNA (6.02%), and small nucleolar RNA (snoRNA; 0.02%) in descending order of abundance (27, 58). mRNA from salivary exosomes can be taken up and translated by recipient cells; this underscores the functional relevance of salivary exosome-mediated RNA transfer (57, 59). The Vesiclepedia (http://www.microvesicles.org) (60) and ExoCarta (http://www.exocarta.org) (61) databases are comprehensive resources for the types of molecular cargos found in extracellular vesicles.

3.2. Structure of Salivary Exosomes

Atomic force microscopy (AFM) and field emission scanning electron microscopy (FESEM) have revealed that salivary exosomes have reversible elastic mechanical properties (57, 62). Specifically, exertion of an outside force causes these exosomes to transition from a spherical morphology to a trilobular structure (Figure 2a,b). Heterogeneity at the surface of salivary exosomes may be due to the presence of CD63 protein in the dense lipid membrane (Figure 2c).

Variations in the nanostructure of salivary exosomes of oral cancer patients compared to healthy individuals may have disease relevance (63). Indeed, cancer-associated salivary exosomes are larger than their normal counterparts (98.3 ± 4.6 nm versus 67.4 ± 2.9 nm; $P < 0.05$) and appear to be derived from multivesicular bodies (MVBs) (Figure 3a,b). Membrane ruptures and elongated nanofilaments surrounding the lumen of MVBs likely contribute to the release of exosomes (Figure 3c-e). There is also a higher density of CD63 on the surface of cancer salivary exosomes.

3.3. Mechanistic Link Between Salivary Exosomes and Systemic Cancer

The discovery of the cancer-specific mutant EGFRvIII mRNA in circulating extracellular vesicles of glioblastoma patients suggested that tumor-derived microvesicles may harbor

disease biomarkers (64). Indeed, cancer-derived exosomes are now known to be a rich source of omic information that reflects the genetic composition and status of their parent tumors. For example, miRNAs 21 and 141 are upregulated in serum exosomes of esophageal squamous cell carcinoma and prostate cancer, respectively (65, 66). Several biomarkers have also been detected in serum exosomes associated with pancreatic cancer, including mutant *KRAS* and *TP53* (67) and the membrane-anchored exosomal protein glypican-1 (68). These findings demonstrate that the utility of tumor-derived exosomes in disease monitoring extends well beyond oral cancer and is likely to be broadly applicable.

Murine models have recapitulated some of the features of human pancreatic cancer and salivary exosome production. For example, mRNAs that originated in orthotopic pancreatic tumor xenografts were found in salivary exosomes (69). Furthermore, the biogenesis of tumor exosomes was suppressed by the introduction of a dominant-negative RAB11 GTPase (DN-RAB11), and this correlated with a reduction in biomarkers that were present in salivary exosomes. This study demonstrated that tumor-derived mRNAs are the cargo of exosomes and reach the salivary gland via the circulation, providing a mechanistic link between salivary exosomes and distal tumors. In subsequent studies using this model, the saliva from tumor-bearing mice was able to suppress the expression of genes associated with the activation of natural killer cells (70). Consistent with its effect on salivary biomarkers, the expression of DN-RAB11 reversed this effect. Thus, salivary exosomes also appear to dampen the immune response to their parental tumors through gastrointestinal tract.

The migration of exosomes from the primary tumor to the salivary glands is not confined to pancreatic cancer models. Orthotopically injected human lung cancer cells expressing a green fluorescent protein (GFP)-tagged cell surface marker (CD63) also gave rise to GFP-positive vesicles in mouse saliva (71). The cargo delivery role of these exosomes was confirmed by the presence of human GAPDH mRNA.

4. ELECTROCHEMICAL BIOSENSORS

The current gold standard methods for isolating exosomes involve ultracentrifugation through a density gradient or sucrose cushion (72). However, these approaches are expensive and laborious. Furthermore, detection of exosome-associated ctDNA in saliva using conventional polymerase chain reaction (PCR)-based methods has largely failed due to its short fragment length and low quantity (73). Therefore, there is a need to develop more practical and efficient methods for the isolation of exosomes, and the quantification of their molecular cargos.

EFIRM is a technology that may meet this need. EFIRM allows quantification of ctDNA via an electrochemical sensor that is activated by capture and detector probes complementary to the ctDNA target (74, 75) (Figure 4). In the first step, pyrrole coating of gold electrodes facilitates the attachment of a single-stranded oligonucleotide capture probe at a surface density of 3.41 molecules/cm^2 (74, 76). The saliva sample is then placed on the electrode in the presence of a cyclic square wave, which opens the hairpin structure (−300 mV, 9 s) of the capture probe and aids hybridization of the negatively charged DNA (+200 mV, 1 s). A complementary biotinylated single-stranded detector probe is then added,

and binding to targets is measured by addition of horseradish peroxidase (HRP) and the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. Redox of the HRP generates a current, which is amplified by TMB-dependent regeneration of HRP. The amount of current is proportional to the concentration of target immobilized on the electrode (77).

EFIRM has been used successfully to detect oncogenic mutations of the epidermal growth factor receptor (*EGFR*) gene in the saliva and plasma of patients with non-small cell lung cancer (73, 78, 79) (Figure 5). Receiver operating characteristic curve analysis demonstrated area under the curve values of 0.94 and 0.96 for *EGFR* exon 19 deletion and *EGFP* L858R, respectively. Capturing and analyzing *EGFR* exon 19 deletion and L858R mutation in saliva are emerging as a complementary technique in liquid biopsy and relevant in early cancer detection, as well as in guiding and managing patients on chemotherapy (80). These findings confirm that EFIRM has sufficient sensitivity to meet the demands of point-of-care testing.

5. FUTURE PERSPECTIVE

The use of salivaomics for cancer detection, diagnosis, and disease monitoring is an exciting prospect. Indeed, the ease with which a biopsy from saliva can be obtained would have a positive impact on patients' quality of life. However, before salivaomics can be successfully adopted in the clinical setting, more work is required to understand how exosomes mediate communication between distal tumors and organs such as the salivary glands. Furthermore, there must be a robust evaluation of the validity of salivary exosome-associated biomarkers. It is also currently unclear whether using salivary exosomes would be more effective than current methods of analysis in oncology such as those involving ctDNA, circulating tumor cells, or exosomal miRNA. On the one hand, mutational analysis of ctDNA does not reveal detailed information about signaling pathways that are active in a particular tumor. In this case, additional salivaomic analysis may provide a more information-rich basis upon which decisions regarding treatment could be made. Analysis of salivary exosomes may also be more representative of the whole tumor when compared to circulating tumor cells, which make up a very small fraction of the malignancy. On the other hand, analysis of exosomal miRNA may be desirable. This is because each exosome will contain a subset of the total cellular miRNA complement due to the randomized encapsulation of miRNAs at the point of vesicle formation.

Techniques for the isolation of salivary exosomes and the quantification of their cargo require further optimization. We suggest that EFIRM technology represents a significant step forward in this regard, because it offers a rapid, robust, and cost-effective way to perform salivary biomarker detection. Continued improvement of EFIRM, together with the development of other rapid biomarker isolation techniques, will lead to earlier detection of disease, more rapid treatment, and reduced morbidity and mortality.

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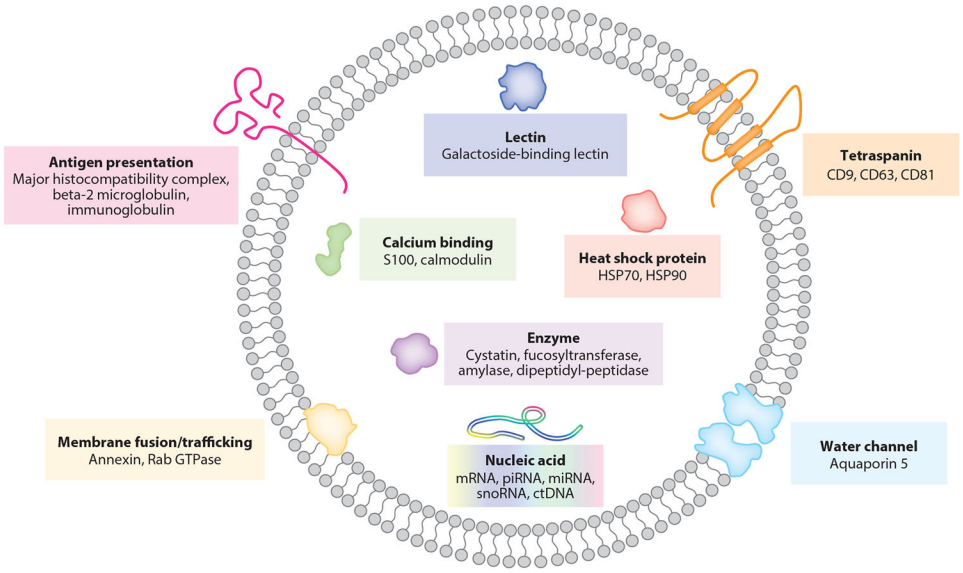

Figure 1.

Structure and contents of typical salivary exosomes. The exosome is surrounded by a phospholipid bilayer, carrying many cell type–specific cargos. Abbreviations: ctDNA, circulating tumor DNA; mRNA, messenger RNA; miRNA, micro RNA; piRNA, piwi-interacting RNA; snoRNA, small nucleolar RNA.

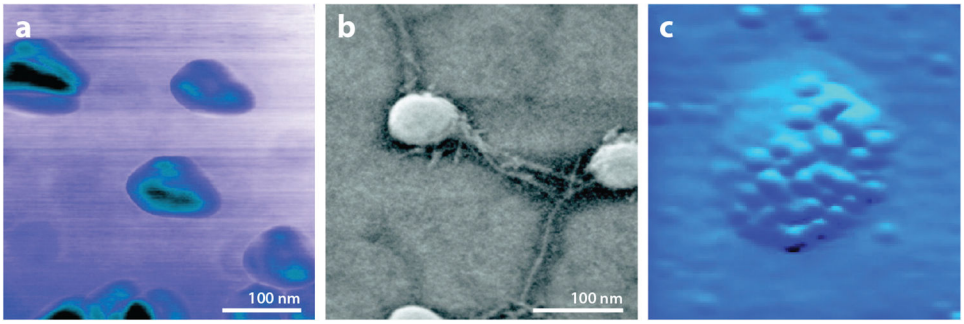

Figure 2.

Nanostructure of salivary exosomes observed under atomic force microscopy (AFM) and field emission scanning electron microscopy (FESEM). (*a*) AFM phase image of salivary exosomes exhibits a trilobular substructure. Surface contrast is presumably attributed to variable constitutive elements on exosomal membrane (e.g., protein and lipid). (*b*) FESEM reveals round-shaped salivary exosomes with intervesicular connections. (*c*) Electron microscopy with anti-CD63 antibody-conjugated gold beads identifies dense tetraspanin molecules on the exosome surface. Figure adapted with permission from Reference 62; copyright 2010 American Chemical Society.

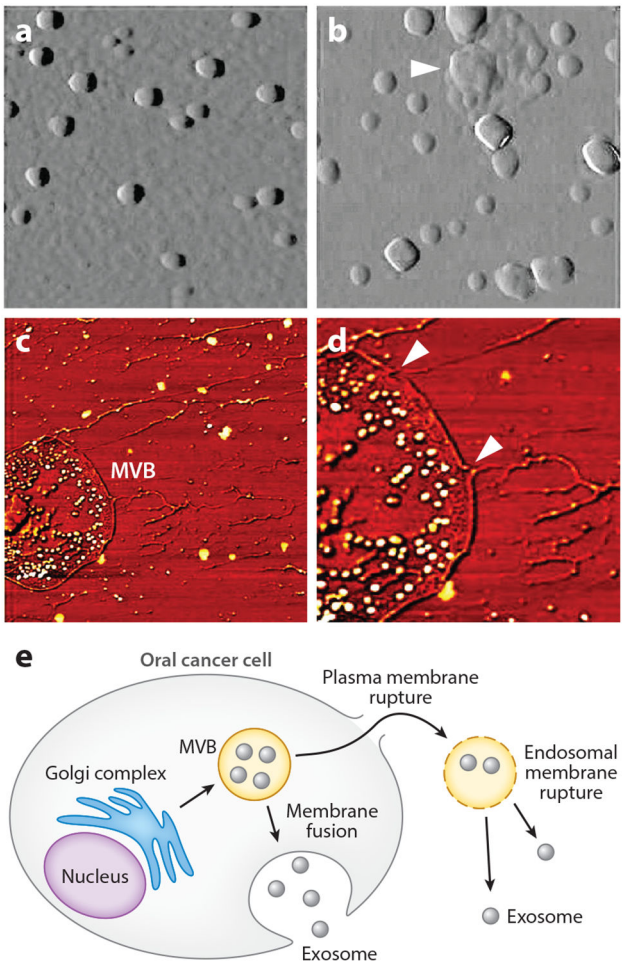

Figure 3.

Exosomes and multivesicular bodies (MVBs) seen in saliva of oral cancer patients. (*a*) Salivary exosomes from healthy donors appear as homogeneous circular structures. (*b*) Salivary exosomes from oral cancer patients show irregular morphology with varying sizes and vesicle aggregation (*arrow*). (*c*) Elongated intervesicular filaments and exosome-like vesicles in MVBs are observed in cancer saliva. (*d*) At higher resolution, membrane ruptures are observed in cancer salivary MVBs (*arrows*). (*e*) Schematic of MVB endosomal membrane rupture and exosome release from oral cancer cell. Figure adapted with permission from Reference 63; copyright 2011 American Chemical Society.

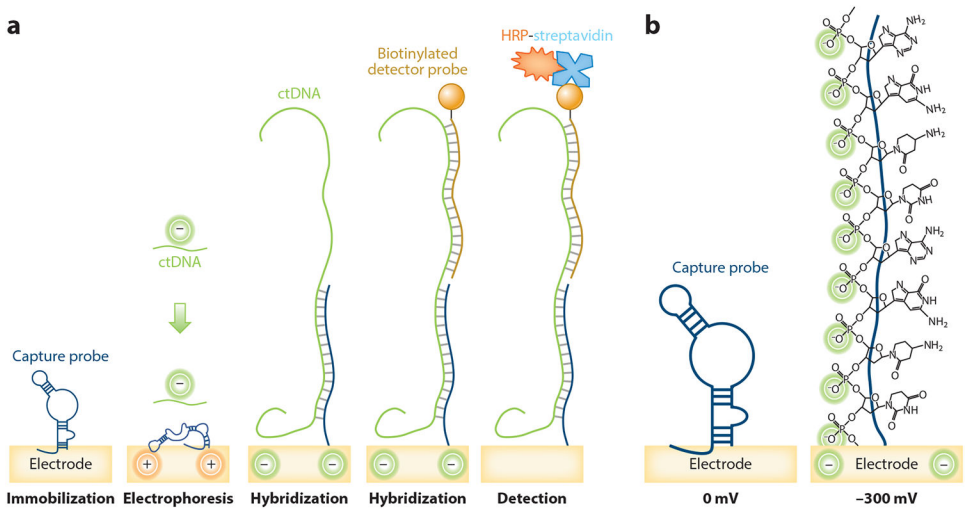

Figure 4.

Schematic of the EFIRM assay. (*a*) Surface preparation: The gold electrodes are precoated with pyrrole and DNA capture probe is immobilized onto the pyrrole-coated electrodes. Electrophoresis and target hybridization: The surface is incubated with the target ctDNA-containing saliva sample and a cyclic square wave electric field is applied at 30 cycles of +200 mV for 1 s and −300 mV for 9 s during hybridization. Detector probe hybridization: A complementary biotinylated single-stranded oligonucleotide detector probe hybridizes with the ctDNA target. Electrochemical detection: HRP-conjugated streptavidin and 3,3′,5,5′-tetramethylbenzidine substrate generate electrical current, which is detected by an electric sensor. (*b*) Steric effect: The negative potential makes a closed hairpin structure of DNA capture probe stretch and form an open structure required for highly efficient intermolecular hybridization. Abbreviations: ctDNA, circulating tumor DNA; EFIRM, electric field–induced release and measurement; HRP, horseradish peroxidase.

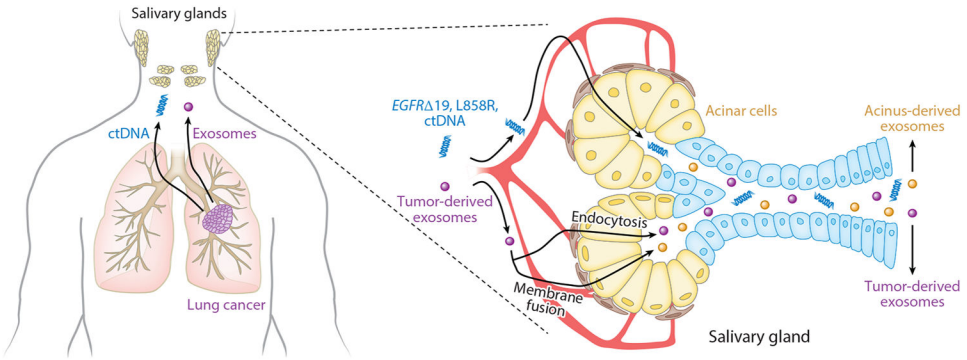

Figure 5.

Saliva-based liquid biopsy for non-small cell lung cancer. ctDNA and tumor-derived exosomes enter the circulation and reach the salivary glands. ctDNA and exosomes are uptaken by salivary gland acinar cells via endocytosis or membrane fusion. Central to saliva liquid biopsy techniques is the capture and analysis of ctDNA, which includes *EGFR* exon 19 deletion and L858R mutation. Combining salivary ctDNA and exosome analyses can provide more comprehensive panels of molecular markers for precision medicine application in a minimally invasive manner. Abbreviations: ctDNA, circulating tumor DNA; *EGFR*, epidermal growth factor receptor.

Table 1

Summary of saliva and plasma ctDNA biomarkers identified in HNSCC. Clinical and laboratory data were retrieved from a database published by Wang et al. (25)

Site	ctDNA	% of positivity (number detected/examined)		
		Saliva	Plasma	Saliva and plasma
Oral cavity	*TP53*	100 (36/36)	85 (11/13)	100 (13/13)
	PIK3CA	100 (2/2)	50 (1/2)	100 (2/2)
	NOTCH1	100 (3/3)	NA	NA
	CDKN2A	100 (2/2)	NA	NA
	Translocation	100 (2/2)	NA	NA
	HPV16 DNA	100 (1/1)	NA	NA
	Total	100 (46/46)	80 (12/15)	100 (15/15)
Oropharynx	*TP53*	80 (4/5)	100 (1/1)	100 (1/1)
	PIK3CA	25 (2/8)	100 (5/5)	100 (5/5)
	FBXW7	67 (2/3)	100 (3/3)	100 (3/3)
	HPV16 DNA	41 (7/17)	92 (11/12)	92 (11/12)
	NRAS	0 (0/1)	0 (0/1)	0 (0/1)
	Total	44 (15/34)	91 (20/22)	91 (20/22)
Larynx	*TP53*	70 (7/10)	86 (6/7)	100 (7/7)
Hypopharynx	*TP53*	67 (2/3)	100 (3/3)	100 (3/3)
Overall		75 (70/93)	87 (41/47)	96 (45/47)

Abbreviations: ctDNA, circulating tumor DNA; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; NA, not applicable.

Table 2

Salivary RNA biomarkers in cancers

Cancer	RNA type	Salivary RNA biomarker	Reference
Breast	mRNA	*CSTA, TPT1, IGF2BP1, GRM1, GRIK1, H6PD, MDM4, S100A8*	30
Esophageal	miRNA	miR-10b, miR-98, miR-144, miR-363, miR-451	81
Head and neck	mRNA	*DUSP1, H3F3A, IL1B, IL8, OAZ1, S100P, SAT*	33
	miRNA	miR-125a, miR-200a	36
Lung	mRNA	*CCNI, FGF19, GREB1, FRS2, EGFR*	32
Ovarian	mRNA	*AGPAT1, B2M, IER3, IL1B, BASP1*	31

Abbreviations: mRNA, messenger RNA; miRNA/miR-, micro RNA.

Table 3

Salivary protein biomarkers for cancers

Cancer	Sample	Salivary protein biomarker	Reference
Breast	Whole saliva	EGF	82
		ERBB2	83
		CA15–3, ERBB2	84
		VEGF, EGF, CEA	85
		CA6	30
		LRP	86
Gastric	Whole saliva	CSTB, TPI1, DMBT1, CALML3, IGH, IL1RA	87
Head and neck	Whole saliva	A1BG, CFB	88
		M2BP, MRP14, CD59, CAT, PFN	89
		FGB, S100, TF, IGHG, CFL1	90
		ADA	91
		IL-8, M2BP, IL-1B	92
	Salivary EVs	A2M, HPa, MUC5B, LGALS3BP, IGHA1, PIP, PKM1/M2, GAPDH	93
Lung	Whole saliva	HP, AZGP1, CALPR	94
	Salivary EVs	Annexin A1, A2, A3, A5, A6, A11, NPRL2, CEACAM1, HIST1H4A, MUC1, PROM1, TNFAIP3	95
Ovarian	Whole saliva	CA125	96

Abbreviation: EV, extracellular vesicle.