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Clinical validity of saliva and novel technology for cancer detection

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Abstract

Cancer, a local disease at an early stage, systemically evolves as it progresses by triggering alterations in surrounding microenvironment, disturbing immune surveillance and further disseminating its molecular contents into circulation. This pathogenic characteristic of cancer makes the use of biofluids such as blood/serum/plasma, urine, tear and cerebrospinal fluids credible surrogates harboring tumor tissue-derived molecular alterations for the detection of cancer. Most importantly, a number of recent reports have credentialed the clinical validity of saliva for the detection of systemic diseases including cancers. In this review, we discussed the validity of saliva as credible biofluid and clinical sample type for the detection of cancers. We have presented the molecular constituents of saliva that could mirror the systemic status of our body and recent findings of salivaomics associated with cancers. Recently, liquid biopsy to detect cancer-derived circulating tumor DNA has emerged as a credible cancer-detection tool with potential benefits in screening, diagnosis and also risk management of cancers. We have further presented the clinical validity of saliva for liquid biopsy of cancers and a new technology platform based on electrochemical detection of cancer-derived ctDNA in saliva with superior sensitivity and point-of-care potential. The clinical utilities of saliva for the detection of cancers have been

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Conflicts of Interests

David Wong is co-founder of RNAmeTRIX Inc., a molecular diagnostic 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. David Wong is consultant to GlaxoSmithKlein, PeriRx, Wrigley and Colgate-Palmolive.

evidenced, but biological underpinning on the existence of molecular signatures of cancer- origin in saliva, such as *via* exosomal distribution, should be addressed in detail.

Keywords

saliva; cancer detection; biomarker; liquid biopsy; circulating tumor DNA (ctDNA); Electric Feld Induced Release and Measurement (EFIRM)

A. Saliva & macromolecular constituents (Salivaomics)

Saliva, a slightly acidic oral fluid (pH=6–7) excreted by the salivary glands, consists of 94–99% water, 0.2% organic and inorganic substances, 0.3% proteins and numerous cellular elements [1, 2], including omics molecular biomarkers present in the blood and urine that can be used in the early detection and monitoring of various types of cancer, such as oral cancer, breast cancer, lung cancer, gastric cancer and pancreatic cancer [3–6]. Multiple proteomic, transcriptomic, and microbiological markers have been identified in saliva that are effective indicators of oral and systemic diseases (Fig. 1) [7, 8]. In addition, saliva testing offers practical advantages of being non-invasive and cost-effective. Therefore, salivary molecular diagnostics is a rapidly advancing field with great potential to significantly benefit public health.

Saliva is produced by three major salivary glands (parotid, submandibular and sublingual) as well as minor salivary glands located throughout the oral mucosa [9]. Saliva production is affected quantitatively and qualitatively by physiological and pathological conditions. Healthy adults generally produce 500–1000 mL of saliva per day at an average flow rate of 0.3–0.4 mL/min [10]. Saliva secretion can be stimulated by smell and taste, psychological and hormonal status, age, drugs, oral hygiene and physical exercise [11]. Each salivary gland secretes a characteristic saliva. For instance, parotid glands produce a serous type of saliva, whereas sublingual glands produce a mucous type of saliva. Saliva can be classified as gland-specific saliva collected directly from individual salivary glands or as whole saliva.

Saliva plays an important role in maintaining the homeostasis of the oral cavity. The function of saliva includes lubrication, buffering, taste, digestion, antibacterial, antiviral, and antifungal protection [12]. The main lubricating compounds in saliva are mucins which are heavily glycosylated glycoproteins [13]. Buffer capacity is mainly attributed to three components of saliva: bicarbonate, phosphate and protein buffer [14]. Enzymes such as carbonic anhydrase contributes to maintaining the pH of saliva, which is important to fight against acids produced by bacteria [15]. Salivary immunoglobulins neutralize viruses and toxins [16]. Another salivary immunity protein lysozyme hydrolyzes bacterial cell wall [17]. The main inorganic components of saliva (calcium, phosphate and bicarbonate) are involved in tooth protection by preventing demineralization and enhancing remineralization of tooth enamel [18]. Saliva facilitates the digestion process by digestive enzyme amylase which breaks down carbohydrates into sugars while lipase initiates fat digestion [19].

The composition of saliva varies between individuals and at different time of day. The most prevalent inorganic components in saliva include sodium, potassium, calcium, magnesium,

chloride, bicarbonate, thiocyanate and phosphate. The organic components comprise uric acid, lactate, immunoglobulins, mucins, lactoferrins, enzymes such as amylases, peroxidase, lipase, lysozyme, kallikreins, hormones such as cortisol, and cytokines [20]. These constituents are released by a cluster of secretory cells called acini in salivary glands into the oral cavity through collecting ducts [1]. The electrolyte composition of fluid secreted by the acinar cells is very similar to that of an ultrafiltrate of plasma [21].

Besides the molecules synthesized in the salivary glands, saliva also includes gingival crevicular fluid, serum transudate, epithelial cells, leukocytes and many microorganisms. As part of the endocrine system, many constituents enter saliva from blood *via* passive diffusion, active transport or extracellular ultrafiltration [22]. Depends partially on the size and partially on the electric charge of the molecule, it can passively diffuse from the capillaries surrounding the salivary glands to the acinus cell. For example, steroid hormones pass relatively easily because they are small in size and composed of fatty acids. Another way is active transport of proteins *via* ligand-receptor binding. For instance, B-lymphocyte cells secrete IgA which gets released into saliva by binding to the IgA receptors present on acinus cells [23]. A third means of transportation of biomolecules from blood into saliva is ultrafiltration. Sulfated steroids, estriol sulfates and neutral steroid hormones migrate through the spaces between acinus and ductal cells. Molecules smaller than 1,900 Da (such as water, ions, and catecholamines) are transferred through the gap junctions between the secretory units [24].

In addition, gingival crevicular fluid, a serum transudate or inflammatory exudate produced by the oral mucosa, also flows into the oral cavity and becomes part of saliva [25]. Previous research has suggested that circulating biomolecules associated with diseases may eventually be transported from the bloodstream into the salivary glands [6, 26, 27]. Therefore, saliva, as a “mirror of the body”, is functionally equivalent to blood in reflecting the physiological and pathological state of the body. As a diagnostic fluid, the collection and sampling of saliva can be done noninvasively with minimal risks of cross-contamination. One limitation of salivary diagnostics has been the low concentrations of analytes in comparison with blood. However, with the advent and development of highly sensitive detection platforms, salivary diagnostics could be a huge breakthrough for cancer screening, detection and monitoring [28].

Comprehensive analysis and identification of the content in human saliva is the first step toward discovering and monitoring salivary biomarkers associated with human health and disease status. The discovery of biomarkers has laid the foundation for personalized medicine. Biomarkers are main focus of current research studies, measured in various human biological fluids. Specifically, as the holy grail of diagnostics is non-invasiveness, saliva is a highly desirable biofluid as its collection is non-invasive, inexpensive and easy to perform. Salivaomics is rapidly emerging focusing on comprehensive profiling of all “omics” constituents such as DNA, RNA, proteins, metabolites, and microbiota. It encompasses genomics, transcriptomics, proteomics, epigenomics, metabolomics and microbiomics [29, 30]. Genomics/Epigenomics studies the biochemical characteristics of DNA, genes and their methylation modifications [30]. The onset and development of malignancy are associated with somatic mutations of tumor-specific DNA, found in saliva,

plasma or other biofluids. Salivary genome and epigenome are assayable by a diverse collection of biomolecular techniques, including methylation array, polymerase chain reaction (PCR) and quantitative PCR (qPCR)-based genotyping [30]. Transcriptomics deals with investigation of RNA, including coding messenger RNAs (mRNAs) and non-coding RNAs such as microRNAs (miRNAs). The current comprehensive transcriptomic methodologies comprise mainly microarray profiling and RNA-Sequencing [31]. Proteomics are methods to examine protein profiles. The most common proteomic technologies include sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional-gel-electrophoresis (2-DE), quantitative mass spectrometry (qMS), Western blotting, enzyme-linked immunosorbent assay (ELISA), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI- TOF-MS) or Raman spectroscopy (RS) [30, 32]. Metabolomics is focused on metabolites and providing deep insights into activation of different metabolic pathways. Metabolomics gives the actual biological state of a sample [33], specifically altered in cancer, that can be detected before appearance of symptoms of a disease, thus enabling early detection of many abnormalities [34]. Currently known analytical techniques for quantifying salivary metabolome encompass proton-nuclear magnetic resonance (H-NMR) spectroscopy, gas chromatography mass spectrometry (GC-MS), high performance liquid chromatography-mass spectrometry (HPLC-MS), ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS), plasma mass spectrometry, and capillary electrophoresis-based mass spectrometry (CE-MS) [35–37]. Lastly, microbiomics highlights the diversification of microbiota structures in the development of oral and systemic cancer [29]. The bacteria may induce carcinogenesis by the production of carcinogenic products, *(i.e.* acetaldehyde), chronic inflammation and interaction with eukaryotic cell cycle and signaling pathways [38, 39].

This review presents the latest advancements in saliva-related studies and addresses the translational values of saliva for the early detection of cancer, the second most common cause of death in the United States [30].

Non-small cell lung carcinoma

The combination of five mRNA biomarkers [cyclin I *(CCNI),* epidermal growth factor receptor *(EGFR),* fibroblast growth factor 19 *(FGF19),* fibroblast growth factor receptor substrate 2 *(FRS2)* and growth regulation by estrogen in breast cancer 1 *(GREB1)]* could differentiate lung cancer patients from control subjects with 93.75% sensitivity and 82.81% specificity [33]. Similarly, three proteomic biomarkers (haptoglobin, zinc-α−2-glycoprotein and calprotectin) have been reported to have the discriminatory power of 88.5% sensitivity and 92.3% specificity in saliva (AUC=0.90) [40].

Pancreatic cancer

Several biomarkers (miR-17, miR-21, miR-181a, miR-181b and miR-196a) were also developed for pancreatic cancer [41], but specifically, the combination of two miRNAs (miR-3679–5p and miR-3679–5p) can be used to distinguish resectable pancreatic cancers [42]. They can detect the changes in the concentration of salivary endogenous metabolites. In addition, the comprehensive metabolite analysis of saliva samples revealed eight metabolites (leucine with isoleucine, tryptophan, valine, glutamic acid, phenylalanine,

glutamine and aspartic acid) that are able of discriminating healthy controls from pancreatic cancer subjects [AUC = 0.993] [35]. In turn, based on the Human Oral Microbe Identification Microarray (HOMIM) and qPCR, Farrell *et al.* demonstrated that the combination of *N. elongata* and S. *mitis* in saliva can distinguish pancreatic cancer patients from healthy subjects (AUC of 0.90 with 96.4% sensitivity and 82.1% specificity) [43]. Interestingly, Torres *et al.* reported similar results related to pancreatic cancer patients by using high-throughput sequencing of bacterial small subunit ribosomal RNA (16S rRNA) gene [44].

Breast cancer

Additionally, salivary levels of protein CA15–3 and lung resistance protein (LRP) could be positively correlated when comparing breast cancer patients to controls [45, 46].

Gastric cancer

In case of the detection of gastric cancer, the combination of three biomarkers (cystatin B, triosephosphate isomerase, and malignant brain tumors 1 protein) could reach 85% sensitivity and 80% specificity [47].

B. Head and neck squamous cell carcinoma (HNSCC)

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide [58] and poses high mortality and morbidity for patients [59]. It is a group of tumors located in oral and nasal cavities, paranasal sinuses, salivary glands, pharynx, and larynx [60]. The most common risk factors include tobacco and alcohol consumption, as well as human papillomavirus (HPV) [61] and Epstein - Barr virus infection [62]. Specifically, HPV is considered to be predictive indicator of better HNSCC response to therapy [63, 64]. In addition, the analysis of molecular biomarkers in saliva can be indicative of HNSCC early diagnosis, prognosis, prediction of treatment response, and early detection of tumor recurrence [65].

B.1. DNA methylation biomarkers

The utilization of a genome-wide DNA methylation platform resulted in uncovering two genes, *HOXA9* and *NID2,* that can be used in early detection and follow-up of patients with oral squamous cell carcinoma (OSCC) [50]. Detection of somatic mutations on TP53, PIK3CA, CDKN2A, HRAS, NRAS, or HPV genes (HPV-16 and HPV-18) in saliva results in identification of OSCC in 100% of patients with oral cancers and in 47–70% of patients with cancers of other locations. Moreover, tumor DNA can be identified in saliva taken from treated patients before developing clinical signs and symptoms of recurrence [66]. Moreover, different panels of methylation markers found in saliva were developed in the literature to detect HNSCC [65]. For example, a methylation panel of DAPK1, p16, and RASSF1A genes can be used to identify HNSCC individuals with an accuracy of 81% (sensitivity of 94% and specificity of 87%) [67], while a panel of KIF1A and EDNRB hypermethylation had 77.4% sensitivity and 93.1% specificity [68]. Significantly, EDNRB methylation in salivary rinses may have potential to identify patients at risk for oral premalignant and

malignant lesions [69], while TIMP3 methylation for detection and monitoring of high risk HNSCC individuals for local recurrence [70].

B.2. RNA biomarkers

In addition, salivary miR-9, miR-191, and miR-134 were found to serve as biomarkers for HNSCC [71]. The decreased expression profiles of miR-125a and miR-200a [51] as well as elevated levels of miR-31 [52] were reported in saliva of OSCC. Specifically, salivary miR-31 can be used to monitor disease. It shows significantly increased levels in patients with oral carcinoma at all clinical stages, but no expression changes in premalignant lesions and healthy controls. Decreased levels of miR-31 were also observed in saliva after excision of tumor [52]. Similarly, the expression level of miR-139–5p was significantly reduced in OSCC samples compared to the controls, and returned to its original state after surgical removal of the primary tumor [72]. In addition, seven salivary RNA biomarker transcripts *(DUSP1, H3F3A, IL1B, IL8, OAZ1, S100P* and *SAT)* were identified to distinguish OSCC from controls [53], while the recent study performed by Horvath *et al.*, showed that salivary IL6 mRNA may serve as a biomarker to diagnose OSCC [54]. Another study reports about elevated levels of transgelin mRNA in saliva of OSCC patients, that is strongly correlated with poorer overall survival [73]. In turn, Tang and colleagues evaluated that salivary MALAT-1 (lncRNA) was present in all the OSCC patients [74].

B.3. Proteomic biomarkers

It was also suggested that the increase in tumor antigen CA15–3 and antibodies for tumor protein markers c-erbB2, CA-125 and p53 in saliva may be used as biomarkers for the oral cavity cancer and other sites [55]. In addition, the elevated levels of IL-10 and IL-13, and decreased levels of IL-1 receptor antagonist (IL-1ra) can be indicative for OSCC [56]. Similarly, overexpression of CD44 soluble (CD44sol) in saliva of laryngeal carcinoma patients before and after surgery were reported compared to healthy controls [75]. A four-protein panel consisting of MMP1, KNG1, ANXA2, and HSPA5, proved high sensitivity (87.5%) and specificity (80.5%) in detection of OSCC [76]. In turn, increased expression of salivary RETN in the OSCC individuals were highly correlated with late-stage primary tumors, advanced overall stage, and lymph node metastasis [77].

B.4. Metabolic biomarkers

In turn, decreased level of valine, leucine, isoleucine, and phenylalanine [36], while elevated levels of porphyrin [57], can indicate oral squamous cell carcinoma (OSCC).

B.5. Microbial biomarkers

Recently, Furquim et al. studied oral microbiome of patients with higher risk of developing OSCC than healthy controls. They observed specific microbial differences associated with gingival disease (Prevotella, Streptococcus, Prophyromonas and Dialister), oral graft-versus-host disease (GVHD) (Firmicutes) and oral mucositis [59].

C. Liquid biopsy based on somatic DNA mutations in circulating tumor DNA (ctDNA)

Liquid biopsy can overcome issues that are associated with tissue biopsy. Screening and monitoring individual's health information can be done by convenient access for biofluids containing molecular signatures released from afflicted tissues. Liquid biopsy can screen and detect the presence of cancerous cells through analyzing cell-free DNA in the circulation. Circulation tumor DNA (ctDNA) is a fragmented tumor DNA. There are many pathologic and normal physiologic mechanisms that can release ctDNA into circulation. Both apoptotic or necrotic tumor cells release fragmented DNA into the circulation [78]. Fragmented circulating free DNA was originally recognized by Mandel and Metais in the blood of healthy individuals in 1948 [79]. In 1977, Leon discovered that cancer patients have increased ctDNA concentration in the circulation [80]. A recent study has found that most of the cell-free DNA fragments are 180 to 200 base pairs (bp) [81–83]. There have been many attempts to utilize blood samples to detect ctDNA, non-invasively provides information regarding tumor genomes [84, 85]. For this reason, ctDNA liquid biopsy is a surrogate for "tissue biopsy". The use of liquid biopsy provides rapid and cost-effective means to detect disease and collect pertinent information regarding therapeutic targets. Clearly, the major advantage of ctDNA compared to tissue biopsy is the noninvasiveness of the collection that enables repetitive, longitudinal molecular analysis of cancer located in body parts that are difficult to access by surgeons and eliminates the risk for potential complications of tissue biopsy. In addition, cfDNA analysis can address the issue of tumor molecular heterogeneity as opposed to single tumor lesion. Even, without the evident clinical signs or symptoms of the disease, the ctDNA enables to diagnose the early onset of the cancer disease [86]. Therefore, clinical applications of ctDNA to cancer treatment are enormous such as diagnosis and molecular profiling, tracking of therapeutic response, monitoring of resistance and tumor heterogeneity, detection of postsurgical residual disease as well as early cancer detection. However, liquid biopsy has also some limitations. The ctDNA assays still need to be more clinically validated before fully entering clinical utility, specifically in early-stage cancer, treatment monitoring, or residual disease detection. The evidence of clinical validity and clinical utility of ctDNA assays is still lacking to be considered useful for cancer screening [87].

C.1. Liquid biopsy for cancer detection

Clinical applications of liquid biopsy are expected to become a valuable tool to apprehend the steps of tumor development. For example, ctDNA fragments, which flow in the bloodstream, can be utilized as tumor genotyping samples as they incorporate exact same genetic defects to those of tumor cells (Fig. 2).

These include point mutations, rearrangements, amplifications, microsatellite instability (MSI), loss of heterozygosity (LOH), and tumor-associated DNA methylation [88]. As shown, this technique will become useful in assisting clinical treatment decision of cancer in individuals. Furthermore, liquid biopsy can be applied to detect indicators/biomarkers of diseases and monitor treatment efficacies including recurrent tumors and onset of acquired resistance mutations.

CtDNA can be used in screening for cancer and tumor detection as it can reveal whether cancer exists or not, even cancer's relapse after treatment (recurrence). Salivary circulating biomarkers can be applied in head and neck cancer [89–91]. Though, Epstein-Barr virus (EBV) DNA in plasma can be useful to screen for early asymptomatic nasopharyngeal cancer in people who do not have any signs or clinical symptoms yet [92]. Liquid biopsies in head and neck cancer is a promising field, particularly with nasopharyngeal carcinoma (NPC) and HPV-positive HNSCCs [91]. Another study evaluated the total concentrations of plasma DNA and *KRAS* mutations in colorectal cancer (CRC) patients [93]. Analysis revealed that more tumor fragments are existing and that increased number of circulating tumor cells (CTCs) is related to the biphasic size divisions of plasma DNA. One way to detect solid tumors is by revealing the reoccurrence of somatic rearrangements. McBride *et al.* depicted genomic rearrangements in 3 cancers and proved that it is possible for PCR assay to find out a copy of tumor gene within the plasma without underlying errors [94]. Also, utilizing blood-based CRC screening test involving *SEPT9* biomarker has been prevalent for diagnostic method. It is possible for the *SEPT9* biomarker to reveal most part of CRCs in all phases and colorectal regions [95].

CtDNA is also utilized as prognostic biomarkers. A study by Lecomte *et al.* investigated the mutations in *KRAS* gene and focused on the cyclin-dependent kinase inhibitor 2A hypermethylation within patients who have CRC. Patients with no trace of ctDNA who have *KRAS* mutations or *CDKN2A* gene promoter hypermethylation were shown to have 100% possibility of 2-year survival rate. And this, in turn, indicated the beneficial role as a prognostic biomarker of the ctDNA to identify the recurrence of CRC within the patients [96]. A study by Diehl *et al.* further demonstrates that patients who had evidence of ctDNA had a relapse of disease within a year after surgery. This study additionally indicated that high concentration of cfDNA and *KRAS* mutants were evidences of the poor result for metastatic CRC patients [97]. ctDNA's role as a predictive biomarker also exhibits many benefits. A study by Diaz *et al.* examined whether *KRAS* mutant DNA is seen within the CRC patients who were getting monotherapy with panitumumab. The result showed that for patients who were receiving the treatment for the duration of 5 −6 months of time, 38% of mutations were detected [98].

Liquid biopsy's most essential and prevalent application is tracking of the response to certain therapies which are identified to have resistance mechanisms. Liquid biopsy is able to avoid the difficulties caused by repeated post-treatment cancer tissue sampling and also show enhanced performance detection of treatment efficacy within many tumor types. Liquid biopsy was demonstrated to be useful in cancer patients when other implements of genomic analysis or repeated tumor biopsy failed [99]. Schwarzenbach *et al.* conducted a study that included 388 patients who had breast cancer prior to chemotherapy and analyzed the LOH in a set of 8 genes [100]. Initially, finding of LOH within ctDNA was first done by Nawroz *et al.* [101], and various studies have been done in other parts of the body. Schwarzenbach *et al.'s* study revealed that increased LOH detection is related with the aggressiveness of breast cancer. Also, ability of liquid biopsy to detect ctDNA methylation is excellent tool to diagnose cancer patients [95, 96, 102, 103]. Another study revealed the correlation between the ctDNA methylation and tumors among esophageal cancer patients [104]. Similarly, taking consideration on studying PD-L1 expression on CTCs in head and

neck cancer, assessment of response to nivolumab or pembrolizumab may affect prognostic results [105]. Studies have also shown correlation between the amount of cfDNA and the progression of cancer. When studied the total of cfDNA in CRC patients during the course of treatment, cfDNA was comparatively higher in the cancer patients than those of the healthy individuals [84, 106]. CtDNA, furthermore, could be used to detect post-surgery residual disease that emerge [107, 108]. In other words, while treatments through surgery can cure and remove major parts of the tumor, one cannot assure whether the treatment worked for each and every patient. CtDNA can serve as an effective marker to detect minimal residual disease which may reoccur.

C.2. Saliva liquid biopsy

The holy grail of early detection of cancer is non-invasiveness and saliva fulfills that. Saliva is a non-invasive biofluid that can be conveniently collected from cancer patients. Saliva is easily accessible and can be indicative of systemic diseases. Therefore, it constitutes a primary fluid for monitoring current health status and for the advancement of point-of-care medicine. Saliva liquid biopsy is a non-invasive mean to detect and evaluate the features of cancer in an individual [28]. Moreover, salivary liquid biomarkers enable real-time monitoring of disease progression and therapeutic responses, initiating the era of personalized medicine [90]. However, in oral cancer, the impact of liquid biopsies in clinical settings is still limited, requiring further studies to discover the best scenario for its clinical use. Tumors may shed cellular material directly into saliva and may reflect pathological changes that occur in distant organ [65]. Circulating markers such as circulating tumor DNA (ctDNA), CTCs, and exosomal miRNAs arouse interest as they opened a new path for new therapeutic methods [89].

CtDNAs of the two front line sensitizing mutations of epidermal growth factor receptor *(EGFR),* L858R and exon 19del, were detected in saliva of NSCLC patients in two blinded clinical studies with 96% concordance with tissue/biopsy genotyping [48, 109] marking the first entry point of saliva liquid biopsy for late stages (III and IV) lung cancer detection. Current technologies of digital droplet PCR (ddPCR) and next generation sequencing (NGS), detect late stages lung cancer with ~80% concordance in plasma.

The superior concordance/sensitivity of saliva liquid biopsy was noted and is currently investigated for its mechanistic rationale, translational and clinical utilities, regulatory evaluations and clinical contexts of uses.

C.3. Current liquid biopsy platforms: ddPCR & NGS

Droplet digital PCR (ddPCR): ddPCR, is an assay utilized to precisely quantify and determine nucleic acid sequences with mutations. The PCR reactions are processed in microscopic scale, each reaction inside isolated space or in water-oil droplets where each amplifying PCR reaction takes place individually. A sample molecule can be fractionated into millions of individual reactions, which enable the technique’s efficiency. An example of advantage of ddPCR shows that utilizing the ddPCR assay for a patient who possessed early stage breast cancer gave detection sensitivity of 93.3% for tumor mutations [110]. While,

utilizing other technique of sequencing gene panel for a patient who possessed a stage 1 non-small cell lung cancer showed ctDNA detection sensitivity of 50% [111].

Next Generation Sequencing (NGS), utilizes the technique of immobilizing DNA pieces in their process of synthesis, and reading their sequence. NGS enables unlimited and enormous number of genes or ctDNA to be efficiently sequenced and in a very short time. CtDNA sequences produced could be compared with the DNA from the same patient's non-disease sources, enabling the identification of changes in the nucleotide of the affected genome compared to the unaffected genome from the same subject/patient. NGS have been advanced to detect gene fusions and other alterations inside the genome. Along with ddPCR, NGS are the most prominent techniques in liquid biopsy.

C.4. Electric Field Induced Release and Measurement (EFIRM) in salivary diagnosis

As the field of liquid biopsy pushes the boundaries of sensitivity in detecting biomarkers, a point of inquiry is the appropriate workflow for mutation detection in clinical settings. Apart from the ability to sensitively identify biomarkers when they are in low abundance relative to wild type DNA, factors such as clinical benefit, sample volume, test turnaround time, and cost effectiveness play roles that must be considered. Electrochemical (EC) sensors have recently found impactful entries in clinical cancer diagnostics [112–114]. The Wong lab at UCLA has developed the "Electric Field Induced Release and Measurement (EFIRM)" technology to specifically capture and monitor in saliva key biomarkers in human cancer patients that can be treated with molecular targeted therapies. The core technology is an electrochemical platform integrating sensitive and specific multiplex assays, optimized for proteomic, transcriptomic and genomic biomarkers in biofluids, including 1) design of nucleic acid probe to specifically amplify electrochemical signals from low number of targets (<10 molecules) without sample extraction and amplification [115, 116]; 2) improve the biocompatibility and probe surface density through conducting polymer interface on electrode [117, 118]; 3) facilitate and enhance the process of incubation through electric waveform [48, 115]. As ctDNA and circulatory biomarkers are now known to be contained in extracellular vesicles (EVs) particularly exosomes, not freely present in the biofluid, the EFIRM technique is further capable to release molecular contents from exosomes using an electric field and rapidly capture the molecular contents present before degradation from constituents of the extracellular biofluid micro-environment [119].

C.4.1. EFIRM liquid biopsy for EGFR mutations in saliva from Lung Cancer patients—

Epidermal growth factor receptor *(EGFR),* a cell membrane receptor with tyrosine kinase activity, is expressed in patients with NSCLC and plays an important role in cellular proliferation, inhibition of apoptosis, angiogenesis, metastatic potential, and chemoresistance [120]. The *EGFR* gene is mutated in the tyrosine kinase (TK) domain in 20% in patients with NSCLC in western countries (50% in Asians) as frontline sensitizing mutations at L858R and exon 19del [120]. These sensitizing mutations are actionable/ druggable by the first-generation tyrosine kinase inhibitor (TKI) erlotinib/genfitinib and more recently by the third generation TKI osimertinib/ADZ9291 [121]. Knowledge of a NSCLC patient's *EGFR* mutation status at these frontline sensitizing mutations and subsequent TKI treatment will confer progression free survival (PFS). The EFIRM

technology detected signature oncogenic *EGFR* mutations in saliva of NSCLC patients, in two blinded clinical studies, with near perfect concordance with biopsy genotyping (96–100%) [48, 109]. In the first blinded study, the AUC for predicting the two specific *EGFR* mutations in lung cancer patients, exon 19del and L858R, were 0.94 and 0.96, respectively [48] (Fig. 4). In the second blinded clinical study, using tumor tissue (surgery/biopsy) genotyping as the gold standard, eLB correctly predicted both EGFR exon 19del and L858R status for all 37 pre- and post- surgery/biopsy saliva NSCLC samples (AUC=1.0) [109].

C.4.2. Exosomal oncogene for pancreatic cancer research by EFIRM in saliva: EFIRM detection of exosome communication of pancreatic cancer-associated exRNA in saliva—Exosomes are lipid microvesicles (30–100 nm) that are able to migrate systemically through the vasculature of the body promoting intercellular communication [122]. They reside in a multitude of biofluids including urine, blood, breast milk, bronchial lavage fluid, cerebral spinal fluids, and saliva (Fig. 5) [123–128]. Although the mechanism is not clear yet, exosomes in body fluids are believed to be closely related to cancer development. However, the current state for exosomal oncogene research is limited by (1) low efficient exosome capture method and (2) no real-time exosome assay.

In a pancreatic cancer animal model, EFIRM was utilized to examine the biofluid compartments that connect the distal tumor (pancreatic cancer), blood and saliva as well as conditioned media from Pan02 cells by selectively capturing exosomes from the biofluids to test the hypothesis that exosomes mediate the systemic dissemination of the tumor-derived biological functions [129]. An exosome-specific EFIRM technology was able to first selectively capture CD63 (exosomal specific membrane protein marker) positive exosomes and then concurrently perform real-time detection for nucleic acids and/or proteins. In this study, EFIRM was able to capture exosomes from saliva, serum and panc02 culture media. In addition, all of the 7 exRNAs were found in exosomes derived from saliva, serum and also panc02 cells. Among them, 6 of the 7 genes were found to be upregulated in both saliva and serum-derived exosomes of tumor-bearing mice when compared to control, while *Foxp1* was found to be significantly upregulated in saliva-derived exosomes, and *Gng2* was found to be significantly upregulated in serum-derived exosomes of tumor-bearing mice. Aside from whole serum, tumor, tumor-derived exosomes, serum-derived exosomes, saliva and saliva-derived exosomes all exhibited upregulation of most, if not all, of the 7 validated pancreatic cancer specific salivary transcriptomic biomarkers.

Concluding remarks and future perspectives: The symptoms of cancer are often not specific until advanced stages of the disease. There is an urgent demand for developing rapid, highly accurate and non-invasive tools for cancer screening, early detection, diagnostics, staging and prognostics. Biofluid-based detection targeting molecular biomarkers of cancers has gained great attentions in clinical community due to its non-invasiveness, potential for early detection through screening of patients at high risk, and validity for cancer monitoring and management. Discovery of cancer-associated biomarkers in biofluids has greatly been benefited from recent advances in high-throughput technology of ‘omics’ analysis, which could allow sensitive and reliable assessment of the level of molecular targets. Plasma has been the primary choice of biofluid for apparent scientific

reasons, but other biofluids such as CSF, urine and saliva have been explored for the feasibility in clinical use as a diagnostic matrix harboring cancer-associated biomarkers. The clinical validity of salivary nucleic acids for the assessment of systemic diseases has lately been demonstrated. This review overviews data reported in the recent literature and discusses the clinical significance and prospects for the application of saliva in the early detection of cancer with the potential for being advanced to translational and personalized medicine. Validity of salivary diagnostics for the detection of cancers, can be successfully correlated with clinical diagnosis and further investigated for use as biomarkers of histological grading and clinical staging of the disease.

For the past years, tremendous efforts have been devoted for the development of cancer-specific biomarkers based on ‘omics’ signatures. Unfortunately, only a few cancer biomarkers have entered routine use. Even fewer have been approved for population screening or diagnosis. For the omics biomarker to be effective, the concentration should sufficiently differentiate from normal during early disease stages and should reflect the extent or severity of the disease. The heterogenous complexity of the pathological state of cancer poses tremendous challenges for the clinical utilization of omics biomarkers, and the various omics technologies still bear technical issues of reproducibility and a high false positive rate. Recently, liquid biopsy on circulating cell free DNA with cancer-associated genomic mutations has become a great interest in cancer detection. There are some inherent issues on the clinical validity of ctDNA-based liquid biopsy to be used for general screening purposes, such as the inability to locate the anatomical origin of ctDNA. Nonetheless, targeting ctDNA with the specificity and sensitivity of detection being measured in binary readouts (presence or absence) could be advantageous over any kinds of omics biomarkers relying on differential levels of target molecules.

To achieve sufficient power in clinical performance and avoid detection of false positives, it is imperative to have a highly sensitive and specific detection platform for ctDNA-based LB. Current practice of LB primarily relies on PCR-based or NGS-based analysis. NGS-based approach has clear advantages including the ability for whole-genome wide profiling of associated mutations, but at the same time it still presents some technical and practical hurdles to be overcome for clinical utilization in clinics, such as required sample processing time, dedicated data analysis, and lack of point-of-care capability, *etc*.

An emerging technology platform with reliable, robust and superior clinical performance in detecting ctDNA will be greatly beneficial to LB-based cancer detection. Current study shows EFIRM’s capability outperforming PCR-based or NGS-based LB and EFIRM’s superior performance in detecting ctDNA is due to a 25-fold higher sensitivity than current technology of ddPCR and the presence of ultrashort fragments of ctDNA in biofluids (Li *et al.,* manuscript in preparation). Scientific rationale for saliva as credible biofluid for cancer detection must be determined. It is imperative to elucidate biological mechanism for the presence of salivary biomarkers associated with cancer and mechanistic details on the distribution of cancer-origin biomolecules into saliva. Recent studies have evidenced the role of exosomes in systemic distribution of disease-specific biomolecules including cancer-specific biomarkers. Quite a few studies have demonstrated exosomal association of salivary biomarkers, which demonstrate the potential role of exosome as a cargo for the transfer of

salivary biomarkers from systemic disease including cancer at a remote anatomical site [130, 131]. A study has also demonstrated exosomal transfer of cancer-origin biomarkers into saliva using animal model of pancreatic cancer [129]. However, more study is needed to advance our understanding on the biogenesis and biodistribution of exosomal biomarkers into saliva. Currently, it still remains to be addressed what the roles of the salivary glands are in this process and if cancer-origin biomarkers are further processed and presented in saliva. It is also intriguing to study if exosomal cancer biomarkers in saliva have any physiological downstream effects. It has been shown that saliva exosomes from tumor-bearing mice modulate immune cell phenotype and anti-tumor cytotoxicity [132]. This finding reveals an important and previously unknown mechanism of antitumor immune regulation and provides new insights into our understanding of the alterations of saliva and salivary exosomes during tumor development.

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Abbreviations

LB	liquid biopsy
cfDNA	cell free DNA
ctDNA	circulating tumor DNA
EFIRM	electric field induced release and measurement
ddPCR	droplet digital polymerase chain reaction
NGS	next generation sequencing

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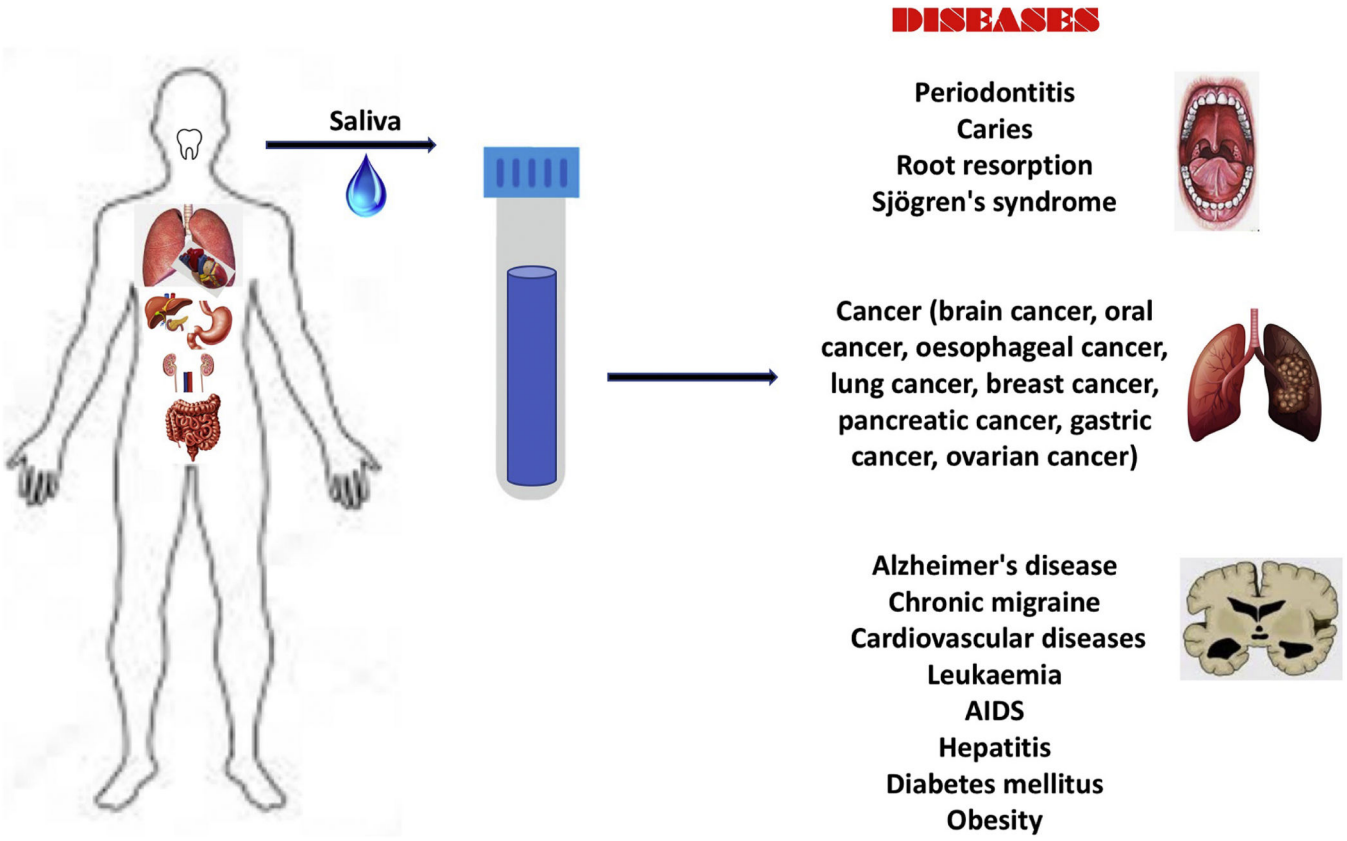

Figure 1.

Clinical utilities of saliva for the diagnosis of local and systemic diseases. Saliva has been proposed or proven to be credential biofluid for detecting variety of systemic abnormalities including cancers.

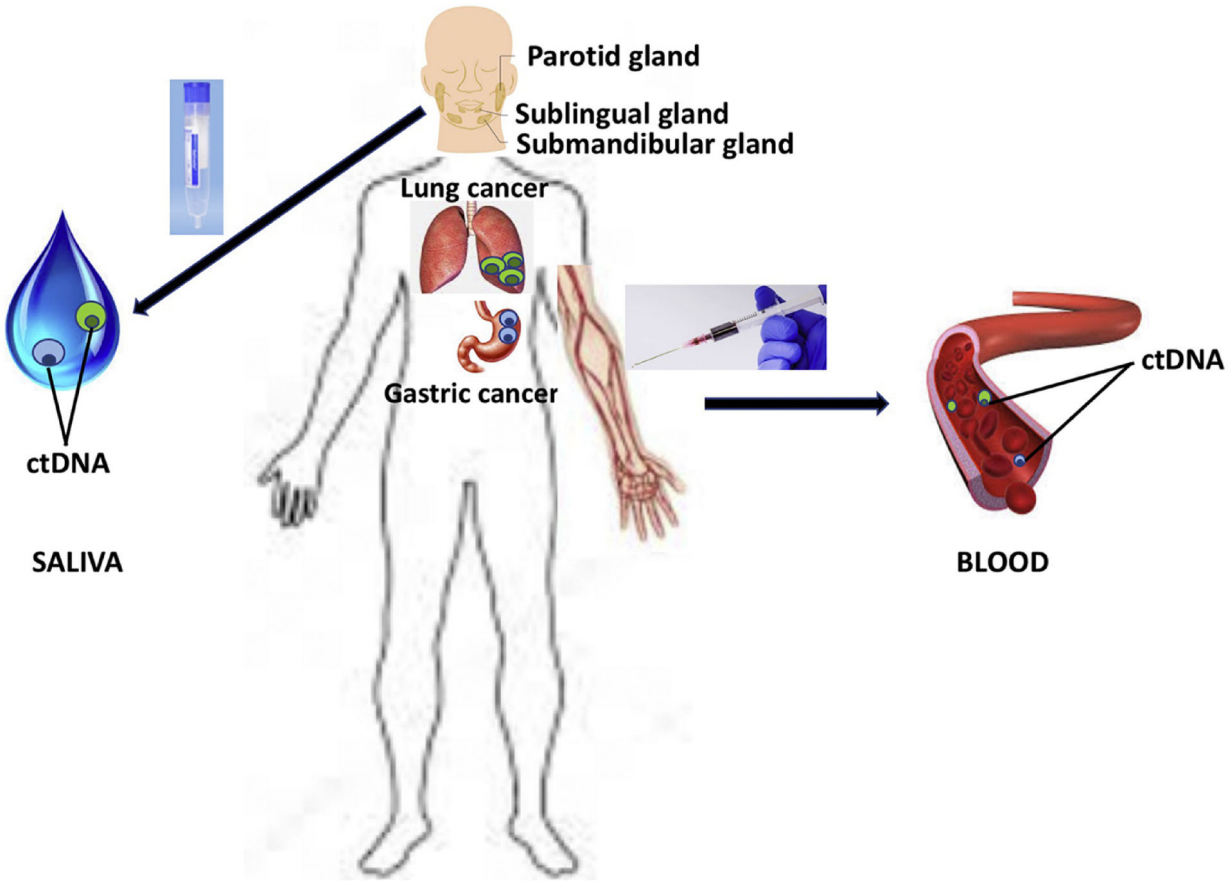

Figure 2.
Liquid biopsy – circulating tumor DNA in blood and saliva.

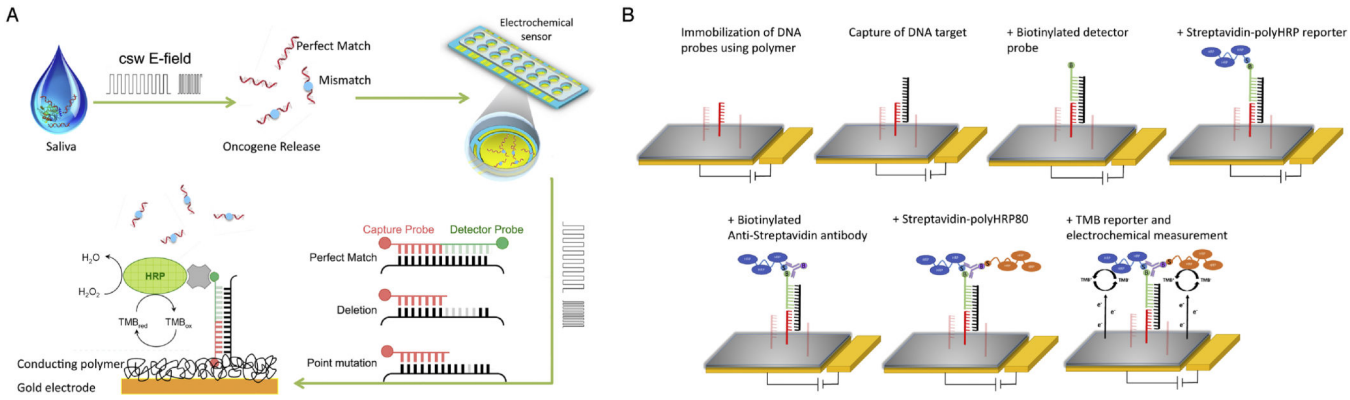

Figure 3.

EFIRM core technology. **A.** Principle of hybridization and detection of ctDNA target by EFIRM. The electrochemical sensor is a conducting polymer-based electrochemical biosensor. Paired probes (capture and detector) are designed for each specific target mutant sequences [116, 117]. Cyclic square wave (csw)-E-field is applied to induce molecular hybridization. (adapted from Wei et al. [48]. Reprinted with permission of the American Thoracic Society. Copyright (c) 2018 American Thoracic Society. Noninvasive saliva-based EGFR gene mutation detection in patients with lung cancer, Am J Respir Crit Care Med, 190 (2014) 1117–112. The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society). **B.** Steps in EFIRM assay: First, a nucleic acid capture probe is immobilized on a gold electrode surface by means of a conducting polymer layer. After hybridization of target DNA sequences in biofluid samples, a biotinylated detector probe that is complementary to the target sequence will be added and incubated. Following incubation of samples and nucleic acid probes, a series of reporter agents are sequentially incubated: The first reporter agent is a streptavidin poly-HRP conjugate, followed by a biotinylated anti-Streptavidin antibody, and finally a Streptavidin-PolyHRP80 conjugate. Each step of the assay requires wash-off using a standard buffer solution (SSC or PBS). Final assay measurement occurs by adding a 3,3’,5,5’-tetramethylbenzidine (TMB) solution and recording oxidation-reduction reactions between HRP enzymes and TMB. The signal readout for the assay is completed in 60 seconds.

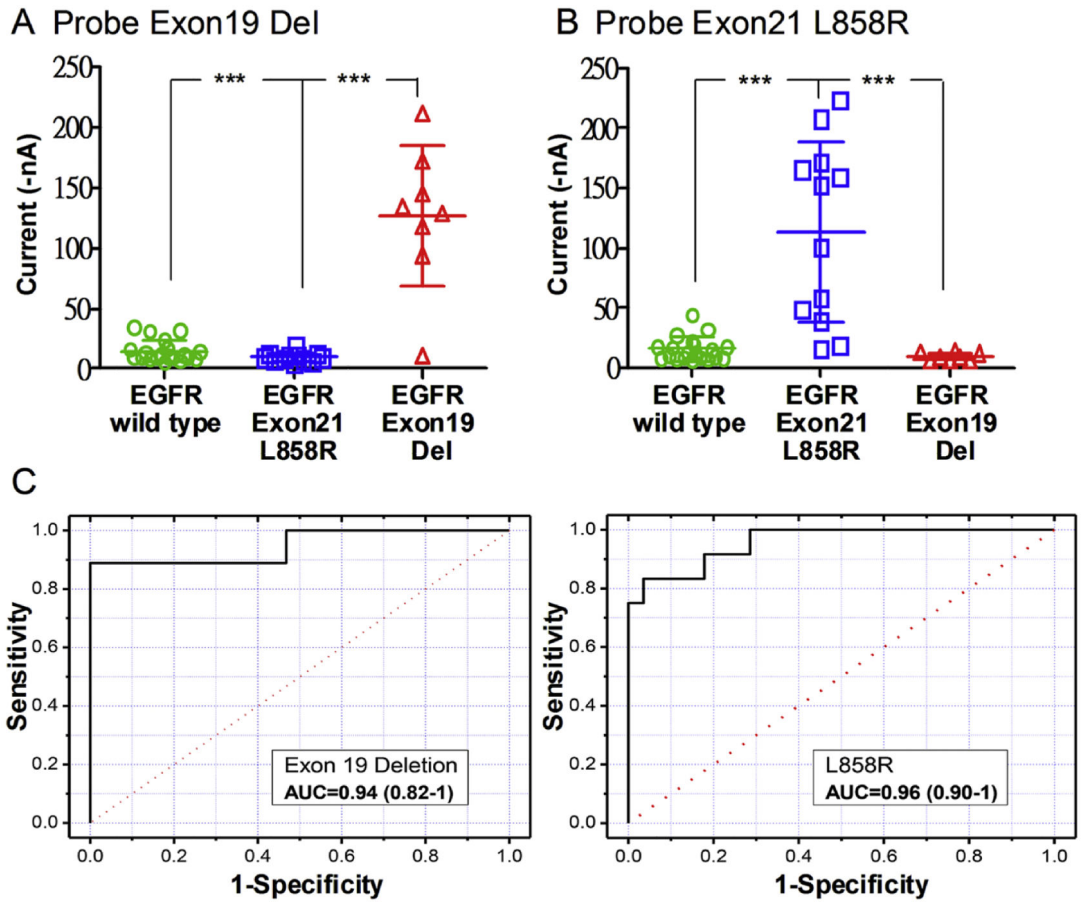

Figure 4.

National Cheng Kung University Hospital in Taiwan (NCKUH): Blinded and randomized detection of EGFR mutations in saliva using EFIRM. The receiver operating characteristic curves for detecting the exon 19 delection [AUC = 0.94, 95% CI, 0.82–1] and the L858R mutation [AUC = 0.96, 95% CI, 0.9–1]. (adapted from Wei et al. [48]. Reprinted with permission of the American Thoracic Society. Copyright (c) 2018 American Thoracic Society. Noninvasive saliva-based EGFR gene mutation detection in patients with lung cancer, Am J Respir Crit Care Med, 190 (2014) 1117–112. The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society).

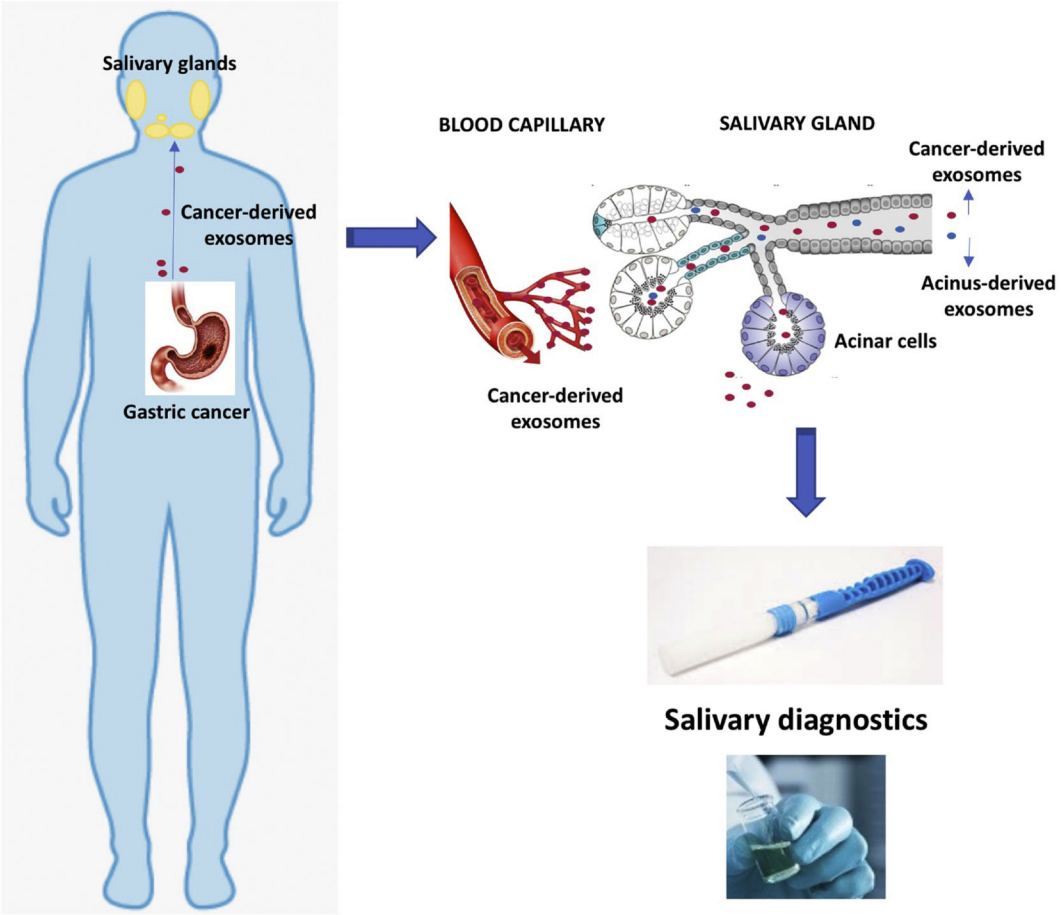

Figure 5.

Proposed route for cancer-derived exosomes from distal tumors to saliva. Exosome transfer from distal tumor through circulation to salivary glands. Two types of exosomes carrying cancer-derived content are released to saliva, either through exocytosis (cancer-derived exosomes) or the cellular plasma membrane fusion with multivesicular bodies (acinus-derived exosomes).

Table 1.

Major salivary molecular constituents.

Salivary constituents	Function
Histatins	anti-bacterial, anti-fungal, buffering, mineralization, wound- closure
Statherins	tissue coating, mineralization, calcium phosphate homeostasis, lubrication, viscoelasticity
Lysozyme	anti-bacterial, innate defense mechanism
Proline-rich proteins	tissue coating, mineralization
Carbonic anhydrases	buffering, maintenance of pH homeostasis
Amylases	anti-bacterial, tissue coating, digestion, dental plaque and caries formation
Peroxidases	anti-bacterial, maintenance of good oral health, prevention of toxic accumulations of hydrogen peroxide (H_2O_2) and inactivation of many carcinogenic and mutagenic compounds
Lactoferrin	antibacterial, antiviral, antiparasitic, catalytic, anti-cancer, and anti-allergic functions, innate defense mechanism
Mucins (mucin 1 & 2)	anti-bacterial, anti-viral, digestion, lubrication, viscoelasticity, tissue coating, non-immune protection of the oral cavity against desiccation and environmental insult
sIgA	immune defense function, mucosal immunity, anti-viral
Cystatins	anti-bacterial, anti-viral, mineralization
Lipase	digestion

Table 2.

Major salivary biomarkers for cancer detection.

Salivary biomarkers	Type of marker	Disease
EGFR [48]	DNA	lung cancer
CCNI, FGF19, FRS2, GREB1 [49]	RNA	
haptoglobin, zinc-a-2-glycoprotein, calprotectin [40]	proteins	
HOXA9, NID2 [50]	DNA	oral squamous cell carcinoma
miR-125a, miR-200a [51], miR-31 [52], DUSP1, H3F3A, IL1B, IL8, OAZ1, S100P, SAT [53], IL6 [54]	RNA	
c-erbB2, CA-125, p53 [55], IL-10, IL-13 [56]	proteins	
valine, leucine, isoleucine, phenylalanine, [36], porphyrin [57]	metabolites	
miR-17, miR-21, miR-181a, miR-181b, miR-196a [41], miR-3679–5p, miR-3679–5p [42]	RNA	pancreatic cancer
leucine with isoleucine, tryptophan, valine, glutamic acid, phenylalanine, glutamine and aspartic acid [35]	metabolites	
N. elongate, S. mitis [43]	bacteria	
cystatin B, triosephosphate isomerase, malignant brain tumors 1 protein [47]	proteins	gastric cancer