Chapter 8
Salivaomics, Saliva-Exosomics, and Saliva Liquid Biopsy

Jordan Cheng, Taichiro Nonaka, Qianlin Ye, Fang Wei, and David T. W. Wong

Abstract The concept of liquid biopsy has emerged into the cancer lexicon to describe detected alterations of tumor biomarkers within body fluids which reflect the presence and the biology of cancer. This is typically performed by assessing circulating tumor cells (CTCs), circulating tumor DNAs (ctDNAs), tumor-derived extracellular vesicles (EVs), microRNAs (miRNAs), and proteins. Although plasma, urine, and cerebral spinal fluid (CSF) are all viable biofluids, growing attention has recently been cast on saliva. Saliva is readily available, can be obtained noninvasively, is easily collected and stored, and also demonstrates compelling pathophysiological association with systemic diseases. Importantly, saliva liquid biopsy delivers the best clinical performance to detect ctDNAs in lung cancer patients. In conjunction with validated biomarkers, and reliable and robust analytical detection tools, saliva has the potential to pioneer a new landscape of real-time point-of-care testing in personalized medicine. This chapter will review the salivaomics, the disease-related biomarker properties of saliva, and the scientific advances in the pathophysiological foundations which make saliva an ideal candidate for noninvasive liquid biopsy. Moreover, a novel liquid biopsy technology termed electric field-induced release and measurement (EFIRM), which works in tandem with the technological advances to extract crucial disease information in saliva, is reviewed.

Keywords Liquid biopsy · Salivaomics · Saliva-exosomics · Biomarker · Cancer · Point-of-care

J. Cheng · T. Nonaka (✉) · Q. Ye · F. Wei · D. T. W. Wong (✉)
Center for Oral/Head and Neck Oncology Research, School of Dentistry, University of California, Los Angeles, Los Angeles, CA, USA

Division of Oral Biology and Medicine, School of Dentistry, University of California, Los Angeles, Los Angeles, CA, USA
e-mail: tnonaka@ucla.edu; dtww@ucla.edu

D. A. Granger, M. K. Taylor (eds.), *Salivary Bioscience*,
https://doi.org/10.1007/978-3-030-35784-9_8

Abbreviations

AUC	area under the curve
CSF	cerebral spinal fluid
CSW	cyclic square wave
CTC	circulating tumor cell
ctDNA	circulating tumor DNA
ddPCR	droplet digital PCR
EFIRM	electric field-induced release and measurement
EV	extracellular vesicle
exRNA	extracellular RNA
HNSCC	head and neck squamous cell carcinoma
HPV	human papiloma virus
HRP	horseradish peroxidase
mRNA	messenger RNA
miRNA	microRNA
MVB	multivesicular body
NGS	next generation sequencing
NSCLC	non-small cell lung carcinoma
OSCC	oral squamous cell carcinoma
PCR	polymerase chain reaction
piRNA	piwi-interacting RNA
TMB	tetramethylbenzidine

8.1 Introduction

Globally, cancer persists as a tremendous burden. It has been projected that 1.7 million new cancer cases will arise in the USA and 609,640 will result in death (Siegel, Miller, & Jemal, 2018). Accordingly, there is an impetus to advance the understanding of cancer biology, diagnosis, and therapeutic strategies. Optimizing the methodologies for screening and early detection of developing lesions before they reach an advanced stage is highly sought after by cancer therapy researchers. For many cancer types, such as non-small cell lung carcinoma (NSCLC), the major subtype of lung cancer, the current gold standard of the traditional tissue biopsy is invasive, and requires that the lesion reaches a critical mass before it is detectable by imaging modalities to warrant a surgical biopsy procedure (Ilié & Hofman, 2016).

An emerging concept, liquid biopsy, holds promise as an alternative to solid tissue biopsy by identifying and detecting alterations in biofluids that may reflect the presence of primary cancerous lesions. As the number of publications involving liquid biopsy increases, the definition continues to evolve. Initially, it referred to the diagnosis and characterization of a solid cancerous lesion by collecting and analyzing CTCs from blood (Chatterjee, 2016). More recently, the definition of liquid biopsy has been extended beyond CTCs in the blood to include other biomarkers that reflect the presence of a tumor. Additionally, liquid biopsy now includes the analysis of other biofluids besides blood. Urine, cerebral spinal fluid (CSF), and saliva are all potential mediums where cancer-associated molecular targets can be found. The

Table 8.1 Considerations of utilizing saliva as a sample type for liquid biopsy

Advantages	Disadvantages
• Saliva contains biomarkers that can be used for clinical liquid biopsy (e.g. ctDNA *EGFR* mutation). • Collection method is easy and noninvasive. • Patients can self-collect repeatedly. • Biomarkers are stable when stored properly (e.g. −80 °C for RNA, protein, ctDNA) (Henson & Wong, 2010).	• Inherent salivary proteins (e.g., amylase) may compete with selected biomarker proteins during analysis (Henson & Wong, 2010). • Production and composition of saliva are influenced by circadian rhythm (Henson & Wong, 2010). Note: inherent to all biofluids. • Patient's unstimulated (basal) and stimulated (during mastication) salivary state affect the tonicity and pH balance of saliva (Chap. 2. By Hernández and Taylor). • Complicated sample processing (e.g., addition of RNase inhibitor and serin protease inhibitor (SUPERase· In™ is a trademark. Aprotinin and PMFS are serine protease inhibitor) are required to protect salivary constituents) (Henson & Wong, 2010).

National Cancer Institute now defines liquid biopsy as "A test done on a sample of blood to look for cancer cells from a tumor that are circulating in the blood or for pieces of DNA from tumor cells that are in the blood" (National Cancer Institute, 2011). While plasma, urine, and CSF are all viable biofluid candidates, more recent attention has been given to saliva. Saliva is readily available (0.5–1.5 l per day), can be obtained noninvasively, is easily collected and stored, and demonstrates compelling pathophysiological association with systemic diseases (Table 8.1).

Saliva is composed of secretions from three major glands (parotid, submandibular, and sublingual) and numerous minor salivary glands located throughout the oral cavity. Secretions from minor glands, gingival cervical fluid, mucosal exudates, microflora, and dislodgements form the oral-esophageal pathway also contributes to the composition (Nonaka & Wong, 2017). The composition of saliva is described in greater detail in Chap. 2 by Hernández and Taylor.

Salivary glands are highly vascularized and are composed of epithelial cells enriched with transporters and channels which provide a conducive environment for molecule exchange between blood and saliva (Gröschl, 2008). Trans blood to salivary pathways of hormones and cytokines have been described using passive and active transport mechanisms through transporters or passage through lipophilic layers of capillaries and glandular epithelial cells (Gröschl, 2008). Due to this proximity to the blood circulation and the predilection for molecule exchange, pathological biomarkers can appear in saliva. Saliva is therefore a practical sample type for liquid biopsy as that reflects systemic health.

8.2 Salivaomics

Salivaomics is the study of saliva and all its -omics constituents, contents, functions, and related techniques. Historically, in 2004, the transcriptome in saliva of stage T1/T2 oral squamous cell carcinoma (OSCC) patients was first examined and

compared with equivalent healthy controls, showing that 1679 extracellular RNAs (exRNAs) exhibited significantly differential expression levels (Li, St. John et al., 2004). Subsequently, a proteomic study showed there was a 20–30% similarity between salivary and plasma proteomes, suggesting that saliva predominantly contains proteins synthesized in the salivary gland along with proteins originating in the blood or lymph sources (Yan et al., 2009). Furthermore, genomic analysis revealed that 30% of salivary exRNAs originate from the host and 70% are derived from oral microbiota (Bonne & Wong, 2012; Looi, Zakaria, Osman, & Jamal, 2012). Analysis of microgram amounts of protein from oral swabs has resulted in over 3700 quantified human proteins (Grassl et al., 2016). These findings provided initial evidence that saliva could be used to differentiate between health and disease and solidified the study of salivaomics as an independent field.

Since saliva is not a homogenous substance, the study of all its components; genomics, epigenomics, transcriptomics, proteomics, metabolomics, and microbiomics are all key components that make up salivaomics. This section will cover the three major—omic groups of salivaomics that are associated with cancer liquid biopsy, RNA (transcriptomics), circulating tumor DNA (genomics), and protein (proteomics).

8.2.1 Salivary Extracellular RNA (exRNA)

Since many different RNA types are present in saliva, the transcriptomic profile of saliva is complex. Saliva contains mRNAs, microRNAs (miRNAs), and other small noncoding RNAs (e.g., piwi-interacting RNAs). Thus far, a major focus in salivary transcriptomic work has been on describing the mRNA and miRNA alterations that differentiate the patients from the healthy subjects (Han et al., 2018; Park, Li, Yu, Brinkman, & Wong, 2006). The salivary transcriptome was first described using high-density microarray technology. This revealed a transcriptomic profile that was highly fragmented with coding and noncoding gene transcripts originating from both the host and oral microbiota (Li, Zhou, St. John, & Wong, 2004; Park et al., 2006, 2007; Spielmann & Wong, 2011).

One of the primary goals of liquid biopsy is to use it as a screening tool for early detection of cancer. Thus, there is merit in discovering viable biomarkers in saliva that signal disease. Transcriptome analysis of saliva from OSCC patients revealed that mRNA biomarkers (*DUSP1*, *H3F3A*, *IL1B*, *IL8, OAZ1, S100P*, and *SAT*) exhibited at least a 3.5-fold elevation in saliva from OSCC patients (Li, St John, et al., 2004). Upon using four of the candidate RNA biomarkers (*IL1B*, *OAZ1*, *SAT*, and *IL8*) and a cutoff of 50% with a logistic regression model, 91% sensitivity and 91% specificity was determined with an area under the curve (AUC) of 0.95.

In the saliva of pancreatic cancer patients, a similar approach was used, and salivary candidate exRNA biomarkers *KRAS*, *MBD3L2*, *ACRV1*, and *DPM1* were deemed associated with the early stage resectable pancreatic ductal

adenocarcinomas. Using these markers, investigators could differentiate pancreatic cancer patients from non-cancer subjects (chronic pancreatitis and healthy control), yielding an AUC of 0.971 with 90.0% sensitivity and 95.0% specificity (Zhang, Farrell et al., 2010).

Similarly, in breast cancer a combination of eight (*CSTA*, *TPT1*, *IGF2BP1*, *GRM1*, *GRIK1*, *H6PD*, *MDM4*, and *S100A8*) salivary exRNAs and one proteomic marker (CA6) could discriminate between saliva samples of patients and control group with 83% sensitivity and 97% specificity (Zhang, Xiao, et al., 2010).

Ovarian cancer salivary transcriptome analysis identified four upregulated and sixteen downregulated exRNAs (Lee, Kim, Zhou, Kim, & Wong, 2012). Using five salivary exRNA biomarkers from the discovery phase (*AGPAT1*, *B2M*, *IER3*, *IL1B*, and *BASP1*) could differentiate healthy controls from patients with 85.7% sensitivity and 91.4% specificity. Using a similar approach, salivary exRNA markers (*CCNI*, *FGF19*, *GREB1*, *FRS2*, and *EGFR*) could be used to separate lung cancer and control with 93.75% sensitivity and 82.81% specificity with an AUC of 0.925.

Another discriminatory salivary exRNA target in salivary liquid biopsy is miRNAs. miRNAs are a class of 21–25 nucleotide long noncoding RNAs that play major roles in the regulation of gene expression and other processes (Ha & Kim, 2014). Recently, miRNAs have been identified in exosomes, membrane-bound vesicles 40–100 nm in diameter, released directly from the plasma membrane (Zhang et al., 2015). Exosomal miRNAs have been shown to regulate oncogenic and tumor suppressor genes (Chen, Liang, Zhang, Zen, & Zhang, 2012). There are vast differences in the miRNA profile between normal cells and cancer cells (Lin & Gregory, 2015).

In an analysis of miRNAs in plasma of head and neck squamous cell carcinoma (HNSCC) patients, elevated levels of miR-21 and miR-24 were detected in plasma from HNSCC patients (Hsu et al. 2012; Lin et al. 2010). Moreover, amplified miR-31 was detected in the plasma of HNSCC patients and was observed to have reduced after tumor resection, suggesting its tumor origin (Liu et al. 2010). Overexpression of miR-106b cluster and underexpression of miR-375 have been associated with HNSCC, and miR-451 appears to resurface during HNSCC recurrence (Hui et al., 2010). Similarly, a high expression of circulating miR-142, miR-186, miR-195, miR-374b, and miR-574 was reported (Summerer et al., 2015).

In the examination of saliva, lower levels of miR-125a and miR-200a were seen in saliva samples of OSCC patients (Park et al., 2009). In one study examining miRNAs for salivary gland tumors, miR-132, miR-15b, miR-140, and miR-223 had a distinguishing ability of 69% sensitivity and 95% specificity with an AUC of 0.90 (Matse et al., 2013). In another study, saliva from patients with esophageal cancer identified miR-144, miR-451, miR-98, miR-10b, and miR-363 as differentiable markers (Du & Zhang, 2017). The novelty in their study was they proposed a regulation network pathway in which the miRNA targets were involved in oncogenesis of the esophageal cancer. miRNAs appear to demonstrate greater stability in saliva compared to mRNAs and thus may be a viable alternative target for salivary liquid biopsy (Gallo, Tandon, Alevizos, & Illei, 2012).

Furthermore, piwi-interacting RNAs (piRNAs), the largest class of small long noncoding RNA molecules (26–31 nucleotides) are present in saliva (Bahn et al., 2015). Their activity has been linked with epigenetic and posttranscriptional gene silencing. Due to their size, piRNAs are able to pass through cell membranes easily and avoid degradation compared to longer RNAs (Han, Li et al., 2017). Salivary piRNAs present another exRNA biomarker source for salivary liquid biopsy.

8.2.2 Salivary Genomics

8.2.2.1 Circulating Tumor DNA

It was reported that blood from cancer patients have higher concentrations of circulating DNA as compared to healthy individuals (Stroun, Anker, Maurice, & Gahan, 1977). Further work from Stroun et al. revealed that DNA could be extracted from the plasma of cancer patients and their source from cancer cells could be determined by strand stability (Stroun et al., 1989). This work shaped and defined the concept of circulating tumor DNA (ctDNA)—the subset of circulating cell-free DNA that can potentially be used as a genetic "fingerprint" for a primary tumor.

ctDNA is believed to originate from tumor cells undergoing apoptosis or necrosis thereby releasing DNA strands into the systemic circulation (Jahr et al., 2001). Pathogenic and physiological processes such as phagocytosis and exocytosis may also contribute (Thierry, El Messaoudi, Gahan, Anker, & Stroun, 2016). Typically, remnant DNA in the blood is degraded by nucleases and eliminated by the liver, spleen, and kidneys (Barra et al., 2015). ctDNA is often described to be roughly 180–200 base pair in length which is characteristic of the apoptotic process and corresponds to the inter-nucleosomal length of DNA that is wrapped around the nucleosome including the linker segment (Diaz & Bardelli, 2014). Recently, it has been shown that ultrashort single-stranded cell-free DNA is present in plasma, which alludes to the possibility that a similar nucleic acid demographic may appear in saliva (Burnham et al., 2016).

A large-scale study on multiple cancer types including HNSCC demonstrated that increasing concentration of ctDNA is associated with advancing stage of disease (Bettegowda et al., 2014). It is not clear if ctDNA promotes carcinogenesis or is merely a by-product of cellular waste disposal as a result of apoptosis. However, there is evidence that ctDNA can promote cancer by transfecting healthy cells (García-Olmo et al., 2010). Laboratory methods for the assessment of ctDNA include allele-specific polymerase chain reaction (PCR), droplet digital PCR (ddPCR), and next generation sequencing (NGS) (Han, Wang, & Sun, 2017). These methods are predominantly PCR-based and have varying strengths and limitations and are mainly used in probing plasma ctDNA. At our laboratory, an emerging electrochemical platform of liquid biopsy named electric field-induced release and measurement (EFIRM) has been developed. This procedure can detect and quantify the ctDNA in saliva of NSCLC patients with superior performance as

compared to the current technologies of ddPCR and NGS (Pu et al., 2016; Wei et al., 2014).

In an intriguing study, the saliva from 93 HNSCC patients was analyzed for human papilloma virus (HPV) DNA and/or somatic mutations related to HNSCC. In patients with tumors in the oral cavity, ctDNAs were detected in saliva with 100% concordance to tissue biopsy as compared to saliva-ctDNA concordance in patients from the oropharynx (47%), larynx (70%), and hypopharynx (67%) (Wang et al., 2015).

8.3 Salivary Proteomics

The saliva proteome is the first salivaomics constituent advocated for salivary biomarker development. An NIDCR/NIH-funded collaborative effort between The Scripps Research Institutes in San Diego, the University of California San Francisco, and the University of California Los Angeles resulting in an annotated catalog of 1166 proteins in salivary proteome of healthy individuals (Denny et al., 2008). This was then deposited into an open access Saliva Proteome Knowledge Base (Ai, Smith, & Wong, 2010). Additionally, a three-dimensional peptide fractionation technique was used to generate a deeper data set including 2340 proteins involved in integral functions in the oral cavity (Bandhakavi, Stone, Onsongo, Van Riper, & Griffin, 2009). Functional analysis shows salivary proteins occupy a higher proportion of metabolic and catabolic processes compared with plasma (Loo, Yan, Ramachandran, & Wong, 2010). This may be a clinical advantage of probing saliva if this particular subtype of proteins is oncogenically relevant (Schulz, Cooper-White, & Punyadeera, 2013).

Saliva proteins such as histatins, statherin, acidic proline rich proteins (PRPs), basic non-glycosylated PRPs are prone to degradation (Helmerhorst & Oppenheim, 2007). Careful considerations must therefore be taken to prevent skewing of downstream analysis of the potential protein biomarkers including other extracellular RNAs and possibly ctDNA that may be present. Preemptive strategies have been developed, published, and curated in order to stabilize the salivary proteins with protease inhibitors and preserve their integrity (Xiao & Wong, 2012).

Presently, the majority of proteomics in cancer biomarkers discovery utilize high-throughput mass spectrometry to identify salivary proteins associated with specific cancers. Breast cancer is a well-studied cancer in terms of salivary proteins. Elevations in EGF (Navarro et al., 1997), c-erbB-2 (Streckfus, Bigler, Dellinger et al., 2000), and CA15-3 (Streckfus, Bigler, Tucci, & Thigpen, 2000) were demonstrated in the saliva of breast cancer patients compared to healthy subjects. Salivary detection of CA15-3 is of particular interest because the same elevation of CA15-3 occurs in serum of patients (Duffy, Shering, Sherry, McDermott, & O'Higgins, 2000). CA15-3 is a transmembrane glycoprotein, frequently overexpressed and glycosylated in cancer. It appears to contribute to cell adhesion involved in metastasis (Duffy et al., 2000). In fact, it has been approved as an

FDA-approved biomarker to track the metastatic progression of breast cancer in serum (Füzéry, Levin, Chan, & Chan, 2013).

Another set of breast cancer salivary biomarker, VEGF, EGF, and CEA was analyzed in saliva of breast cancer patients by testing their predictive power individually or in combination revealing that VEGF and EGF leading to a combined 83% sensitivity and 74% specificity with an AUC of 0.84 (Brooks et al., 2008). CA6, another salivary candidate protein, demonstrated the ability to differentiate between cancer and healthy controls in saliva (Zhang, Xiao, et al., 2010). Lung resistant protein in saliva was observed to present at higher concentrations in Stage 1 breast cancer patients exhibiting the ability to detect the disease at its early stages (Wood & Streckfus, 2015). Liu et al. using 9 candidate salivary lectins with alterations in salivary glycopatterns can predict the presence of Stage 1 breast cancer with an accuracy of 0.902 in a double-blind cohort (Liu et al., 2018).

Salivary proteomic biomarker development for OSCC revealed that M2BP, MRP14, CD59, catalase, and profilin were associated and with a regression model which achieved 90% sensitivity and 83% specificity with a predication rate of 85% (AUC = 0.93) (Hu et al., 2008). In other studies IL-8, M2BP, and IL-1B were discovered as viable biomarkers. IL-8 and M2BP, however, showed better statistical distinction compared to control groups (Elashoff et al., 2012; Hu et al., 2008; Li, St John, et al., 2004). Analysis of the whole saliva from three HNSCC patients with LC–MS/MS revealed alpha-1-B-glycoprotein and complement factor B as unique proteins in cancer (Ohshiro et al., 2007). Additionally, using 2D DIGE analysis and subsequent mass spectrometry showed that beta-fibrin, S100 calcium-binding, transferrin, immunoglobulin heavy chain constant region gamma, and cofilin-1 were increased in HNSCC patients (Dowling et al., 2008). Studies in tongue cancer patients revealed that salivary adenosine deaminase activity may be a good differentiator between healthy and control subjects (Rai, Kaur, Jacobs, & Anand, 2011). Another group utilized selected reaction monitoring (SRM) tandem mass spectrometry to observe that C1R, LCN2, SLPI, FAM49B, TAGLN2, CFB, C3, C4B, LRG1, and SERPINA1 proteins are elevated in the saliva of OSCC patients and that, in particular, CFB, C3, C4B, SERPINA1, and LRG1 were associated an increased risk for developing OSCC (Kawahara et al., 2016).

Analysis of lung cancer saliva samples revealed that three proteins (HP, AZGP1, and CALPR) were higher in lung cancer patients compared to healthy controls. Logistic regression analysis of the diagnostic screening potential for theses biomarkers elicited a result of 88.5% sensitivity and 92.3% specificity (AUC = 0.90) (Xiao et al., 2012). Recently, using an exosome and microvesicle isolation approach coupled with LC–MS/MS-based label-free quantification showcased that four salivary exosome/microvesicle associated proteins (BPIFA1, CRNN, MUC5B, and IQGAP) were dysregulated in lung cancer patients (Sun et al., 2018).

In gastric cancer, four salivary proteins (*mass-to-charge ratio (m/z)* 1472.78, 2936.49, 6556.81, and 7081.17) identified by mass spectrometry appeared differ between gastric cancer and control groups (Wu, Wang, & Zhang, 2009). Xiao et al. identified 519 proteins in the saliva of gastric cancer patients and the data suggest that 48 proteins demonstrated a significantly different gastric profile in gastric cancer

patients (Xiao et al., 2016). From these 48, six proteins (CSTB, TPI1, DMBT1, CALML3, IGH, and IL1RA) were selected as a gastric cancer screening verified with ELISA demonstrating downregulating. Regression model analysis depicted that by using three proteins as a screening protocol (CTSB, TPI1, and DMBT1) would have an 85% sensitivity and 80% specificity (AUC = 0.930).

For ovarian cancer, CA125 is elevated in the saliva discriminating between malignant and benign groups with an 81.3% sensitivity (Chen, Schwartz, & Li, 1990). More recently, 25 overexpressed and 19 underexpressed ($p < 0.05$) proteins between healthy controls and cancer patients were uncovered by fluorescence-based 2D-DIGE coupled with matrix-assisted laser desorption/ionization-time of flight Mass Spectrometry. Discriminatory candidate salivary proteins Lipocalin-2, indoleamine-2, 3-dioxygenase1 (IDO1), and S100A8 were identified and validated using western blotting and ELISA and when validated with 40 ovarian cancer patients and 40 control patients revealing a combined sensitivity of 87.5% and specificity of 86.7% with an ROC of 0.93 (Tajmul et al., 2018).

These proteomic association studies show the vast potential for cancer prediction and that salivary protein can provide for translational applications. Further work must be performed to validate these candidate proteomic biomarkers in prospective clinical trials adherent to the PRoBE (prospective-specimen-collection and retrospective-blinded-evaluation) design in a specific clinical context of use (Pepe et al., 2001).

8.4 Biological Mechanisms of Salivary Exosomes

Recent exploration of salivary genomics, transcriptomics, and proteomics have uncovered associated respective biomarkers source with disease phenotypes. Yet, little is known of the pathophysiological relationship with the distal cancer. How these biomarkers biologically arrive in the saliva is largely unknown. For diseases like pancreatic, gastric, and ovarian cancers, since the neoplastic lesion is geographically distant from salivary glands, it is unclear if the biomarkers that are detectable in the saliva are a by product of the tumor or a specific component of the pathophysiological process.

One mechanism in which these biomarkers may migrate/traffic into the saliva is through extracellular vesicles (EVs) transportation. Traditionally, EVs are organized and distributed into three subgroups based on size: exosomes, microvesicles, and apoptotic bodies (Kalra et al., 2012). Exosomes range from 30 to 100 nm in size and 1.13–1.19 g/mL in density (Théry, Amigorena, Raposo, & Clayton, 2006) and were first isolated from saliva in 2008 (Ogawa, Kanai-Azuma, Akimoto, Kawakami, & Yanoshita, 2008). Traditionally, exosomes are isolated through density gradient or sucrose cushion by ultracentrifugation at 100,000 × g (Théry et al., 2006). Additional methods, however, such as polymer-assisted precipitation (Niu, Pang, Liu, Cheng, & WSB, 2017), immunoaffinity-based capture beads (Sharma et al., 2018),

immunoaffinity-based microfluidic chips (He & Zeng, 2016), and acoustic fluidic chips (Wu et al., 2017) have surfaced with promising capabilities.

In order to reduce the aforementioned variabilities of saliva during collection, it may be ideal to focus on studying the isolated EVs secreted by cancer cells present in the saliva. The term *saliva-exosomics* is used to describe the integration of various -omics approaches (e.g. genomics, transcriptomics, or proteomics) to examine the presence and function of salivary exosomes and exosome-related biomarkers that emerge in the setting of oral and systemic diseases (Nonaka & Wong, 2017).

Sharma, Gillespie, Palanisamy, and Gimzewski (2011) showed direct release of exosomes from oral cancer cells in saliva. In pursuit of the mechanistic tumor salivary exosomal axis, an orthotopic pancreatic cancer mouse model was developed by injecting a pancreatic cancer cell line (Panc02) into the pancreas of a syngeneic mouse (Lau et al., 2013). It was observed that exosome biogenesis could be inhibited by stable transfection of the dominant-negative form of GTPase Rab11 (DN-Rab11). When exosome biogenesis is suppressed by knocking down Rab11 in the Panc02 cells, the salivary transcriptome signature of mice injected with those genetically modified cells was ablated, compared to control tumor-bearing mice. This suggests a mechanistic link between pancreatic exosome biogenesis and the transportation/trafficking/migration of the exosomes to salivary glands and presentation of the disease exRNA profile in saliva.

Yang et al. developed a human lung cancer cell (H460) model expressing hCD63-GFP (an exosome marker) by injecting H460 cells into the chest cavity of immunocompromised mice (Yang, Wei, Schafer, & Wong, 2014). Human GAPDH mRNA was identified in hCD63 + GFP+ exosome-like microvesicles in saliva of the tumor-bearing mice. This finding suggests that human tumor cell-specific mRNA encased in exosome-like microvesicle can be transported from the organ of pathology (lung) to the salivary gland and into saliva.

More recently, Katsiougiannis et al. used a rodent model to show that saliva possess immunoregulatory properties (Katsiougiannis, Chia, Kim, Singh, & Wong, 2017). Saliva from Panc02-injected mice was collected and orally administered into non-tumor bearing control mice. NK cell activation markers, CD69 and NKG2D, were shown to significantly decrease when tumor saliva was gavaged into non-tumor bearing mice. Contrastingly, when saliva collected from mice injected with exosome biogenesis-suppressed Panc02 cells (DN-Rab11 transfected) was orally administered to non-tumor bearing mice, the NK activation markers were not affected. These findings depict an immunological relevance to exosome migration from the primary tumor site.

These physiological studies begin to shine light on the relevance of salivary exosomes and their capability to alter and regulate remote sites in the body (Fig. 8.1). Saliva-exosomics presents a new landscape and new horizon of saliva biology that is just being explored.

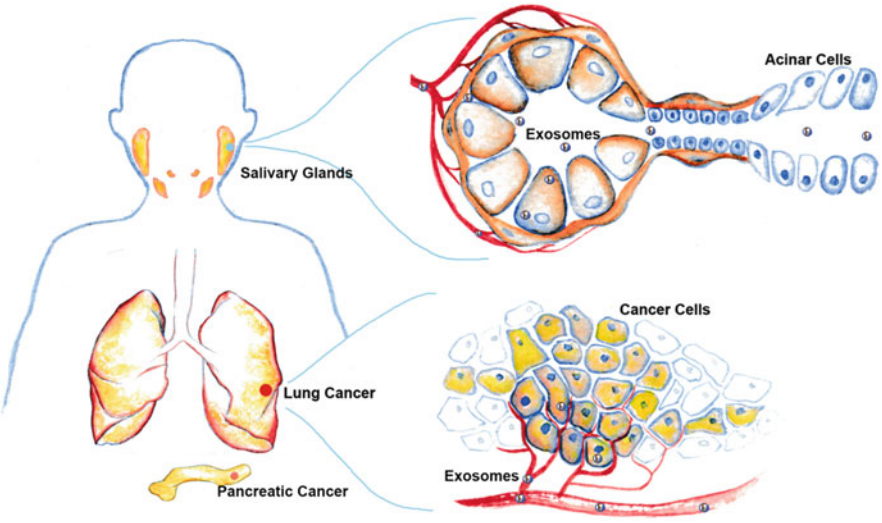

Fig. 8.1 Potential biological mechanism for exosomes trafficking between salivary gland and distal tumor sites. Exosomes (not drawn to scale) are released by distant cancer cells (e.g., in the lung or pancreas) through multivesicular body (MVB) and enter the circulation. Exosomes may be up taken into salivary gland cells through endocytosis or membrane fusion and later released into the saliva. Exosomes have been shown to contain cancer-derived miRNAs, mRNAs, genomic DNAs, and proteins which can be isolated and assayed in saliva

8.5 EFIRM and Saliva Liquid Biopsy

An impactful engagement of saliva liquid biopsy is in the intensely research landscape of liquid biopsy to detect actionable mutation in biofluids, where lung cancer is the major organ site being explored where three actionable mutations (L858R, Exon 19 deletion, and T790M) in the epidermal growth factor receptor (*EGFR*) gene can be drugged and impact on the progress free survival of NSCLC patients (Wei, Yang, & Wong, 2013).

Our group has developed a novel liquid biopsy technology termed electric field-induced release and measurement method (EFIRM). This non-PCR-based electrochemical platform utilizes a capture probe that is complementary to a ctDNA target is designed and then immobilized on the surface of a gold electrode by encapsulating it in a conducting polymer matrix. After the immobilization of the capture probe on the surface of the electrode, the saliva specimen is placed on the surface of the electrode and a cyclic square wave (CSW) is applied. This CSW is designed to specifically lyse the exosomal structure that encapsulates the ctDNA sequence and aids in the DNA hybridization process. EFIRM can disrupt exosomes to release mRNAs and proteins in a similar manner as triton lysis (Wei et al., 2013). Following the incubation of the target sequence to the capture probe, a detector probe that is also complementary to the ctDNA is hybridized. This detector probe is biotinylated at its terminal end, which is then complexed to a streptavidin-horseradish peroxidase

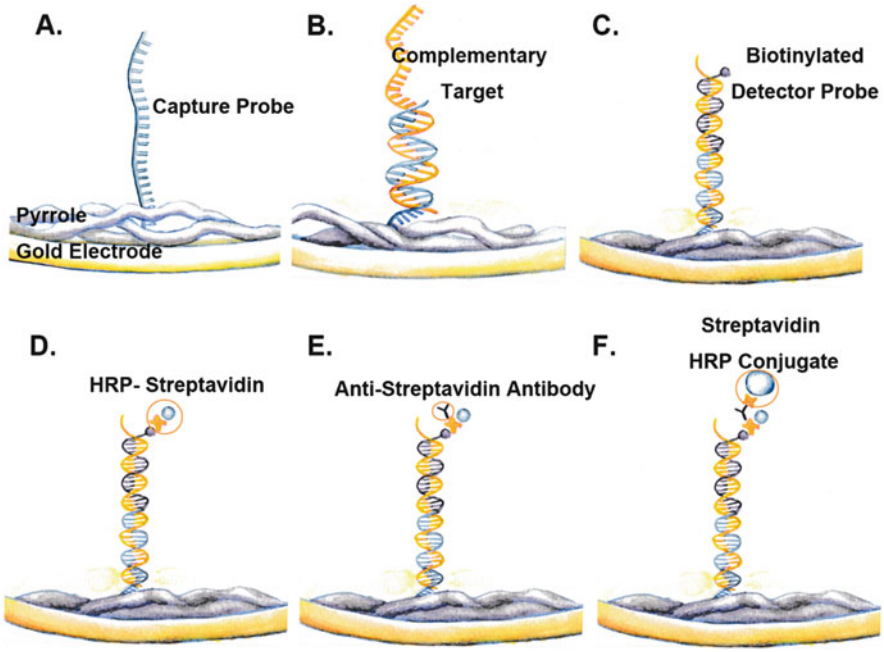

Fig. 8.2 Schematic steps of the EFIRM Assay. (**a**) An electric field is applied polymerizing pyrrole in order to embed a capture probe specific for a cancer biomarker onto a gold electrode. (**b**) Complementary biomarker target is added and hybridizes with capture probe. (**c**) Complementary biotinylated detector probe hybridizes with target. (**d**) HRP (Horseradish peroxidase)-conjugated streptavidin binds to biotin on detector probe. (**e**) and (**f**) A subsequent layer of anti-streptavidin antibody and streptavidin-HRP amplify the signal. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate is added to generate a current through a reaction with HRP. The current is read by the gold electrode which represents the relative biomarker abundance

(HRP). The final output signal is determined by measuring a current generated by a tetramethylbenzadine-HRP reaction which proportionally reflects the amount of detector probe and target present in the saliva sample (Fig. 8.2).

Current clinical practice to detect signature *EGFR* ctDNA for NSCLC is ddPCR and NGS with performance ranges from 60–80% concordance with biopsy genotyping (Cohen et al., 2018; Newman et al., 2014; Phallen et al., 2017). The EFIRM technology detected signature oncogenic *EGFR* mutations in plasma and saliva of NSCLC patients, in two blinded clinical studies, with near-perfect concordance with biopsy genotyping (96–100%) (Pu et al., 2016; Wei et al., 2014).

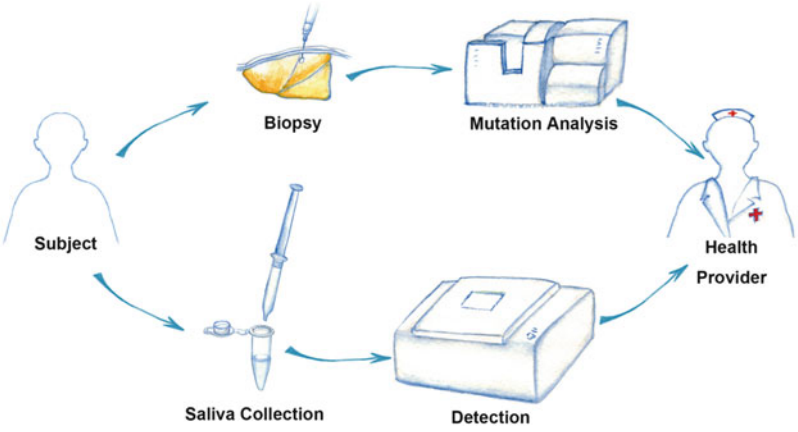

Fig. 8.3 Potential of liquid biopsy in clinical practice. Sampling of biofluids for liquid biopsy can provide monitoring of patient disease progression or efficacy of pharmaceutical interventions. Point-of-care monitoring of liquid biopsy provides clinical information to the health provider with lower turn around time compared to traditional invasive tissue-biopsy and mutation analysis

8.6 Consideration and Future Outlook

Scientific and translational advances have established saliva as a sample type for omics-based biomarker development and liquid biopsy applications. Salivaomics and saliva-exosomics have mechanistically connected salivary glands with networking of personalized omics constituents from systemic origins. At this time, while many discovered salivary biomarkers remain to be definitively validated, it is foreseeable that as reliability and robustness of salivary biomarkers continue to be strengthened and their biological relevance uncovered, saliva liquid biopsy will become a major factor in many clinical applications.

Liquid biopsy will be a valid drug selection tool, treatment monitoring, and detection of acquire resistance mutations in cancer patients. In HNSCC, analyzing both saliva and plasma may be optimal for effective screening and monitoring of cancer (Nonaka & Wong, 2018). In NSCLC, detecting (L858R and Exon19 deletion) ctDNA targets with EFIRM could potentially be used to guide therapeutic decisions for specific tyrosine kinases inhibitors (Wei et al., 2014). The two-mutation assay has been shown to be feasible and therefore expanding the number of targets is needed. For example, T790M, another *EGFR* mutation, is responsible for 50% of acquired resistance to first-generation tyrosine kinase inhibitors if detected, its presence would indicate the use of a third-generation osimertinib (Inal, Yilmaz, Piperdi, Perez-Soler, & Cheng, 2015). Recently, patients been presenting ostimertinib resistant-associated C797S mutation which at this time has no therapeutic options (Wang, Tsui, Liu, Song, & Liu, 2016). Accordingly, if salivary liquid biopsy detects the levels of different mutations (e.g. L858R to T790M to C797S), it can monitor the progression of disease.

Additionally, salivary liquid biopsy can be a viable tool for high-risk population screening. Due to the ease of use and noninvasive nature it may be possible to screen a large population for the possibility of disease. This screening information will lead to subsequent examinations and diagnostic tests that can pinpoint the exact nature of the disease sooner compared to traditional invasive tissue-biopsy and mutation analysis (Fig. 8.3).

The future outlook is promising, however, crucial tasks in the future will be to verify, validate biomarkers, and elucidate the biological interaction between primary tumors and their presence in the salivary milieu. Many biomarkers will need to undergo large-scale prospective double-blinded clinical trials to be definitively validated in a clinical context of uses. In parallel, establishing and developing robustness novel technologies will be essential to bringing salivary liquid biopsy to the forefront of modern medicine.

Acknowledgments This work was supported by Grants (UH2/UH3 CA206126; U01 CA233370; UO1 DE017790) from the National Institutes of Health (NIH) to David T. W. Wong and the Canadian Institute of Health (CIHR) Doctoral Foreign Student Award and Tobacco Related Disease Research Program (TRDRP) Predoctoral Fellowship to Jordan Cheng.

Disclosures David T.W. Wong is a consultant to GlaxoSmithKlein, Wrigley and Colgate-Palmolive.

References

Ai, J., Smith, B., & Wong, D. T. (2010). Saliva ontology: An ontology-based framework for a salivaomics knowledge base. *BMC Bioinformatics, 11*, 302. https://doi.org/10.1186/1471-2105-11-302

Bahn, J. H., Zhang, Q., Li, F., Chan, T. M., Lin, X., Kim, Y., . . . Xiao, X. (2015). The landscape of microRNA, Piwi-interacting RNA, and circular RNA in human saliva. *Clinical Chemistry, 61*, 221–230. https://doi.org/10.1373/clinchem.2014.230433

Bandhakavi, S., Stone, M. D., Onsongo, G., Van Riper, S. K., & Griffin, T. J. (2009). A dynamic range compression and three-dimensional peptide fractionation analysis platform expands proteome coverage and the diagnostic potential of whole saliva. *Journal of Proteome Research, 8*, 5590–5600. https://doi.org/10.1021/pr900675w

Barra, G. B., Santa Rita, T. H., Vasques J de, A., Chianca, C. F., Nery, L. F., & Santana Soares Costa, S. (2015). EDTA-mediated inhibition of DNases protects circulating cell-free DNA from ex vivo degradation in blood samples. *Clinical Biochemistry, 48*, 976–981. https://doi.org/10.1016/j.clinbiochem.2015.02.014

Bettegowda, C., Sausen, M., Leary, R. J., Kinde, I., Wang, Y., Agrawal, N., . . . Diaz, L. A., Jr. (2014). Detection of circulating tumor DNA in early- and late-stage human malignancies. *Science Translational Medicine, 6*, 224ra24. https://doi.org/10.1126/scitranslmed.3007094

Bonne, N. J., & Wong, D. T. (2012). Salivary biomarker development using genomic, proteomic and metabolomic approaches. *Genome Medicine, 4*, 82. https://doi.org/10.1186/gm383

Brooks, M. N., Wang, J., Li, Y., Zhang, R., Elashoff, D., & Wong, D. T. (2008). Salivary protein factors are elevated in breast cancer patients. *Molecular Medicine Reports, 1*, 375–378.

Burnham, P., Kim, M. S., Agbor-Enoh, S., Luikart, H., Valantine, H. A., Khush, K. K., & De Vlaminck, I. (2016). Single-stranded DNA library preparation uncovers the origin and diversity of ultrashort cell-free DNA in plasma. *Scientific Reports, 6*, 27859. https://doi.org/10.1038/srep27859

Chatterjee, D. (2016). Liquid biopsies: Handle with care. *Journal of Pathology and Laboratory Medicine, 1*, 3–6.

Chen, X., Liang, H., Zhang, J., Zen, K., & Zhang, C. Y. (2012). Secreted microRNAs: A new form of intercellular communication. *Trends in Cell Biology, 22*, 125–132. https://doi.org/10.1016/j.tcb.2011.12.001

Chen, D. X., Schwartz, P. E., & Li, F. Q. (1990). Saliva and serum CA 125 assays for detecting malignant ovarian tumors. *Obstetrics and Gynecology, 75*, 701–704.

Cohen, J. D., Li, L., Wang, Y., Thoburn, C., Afsari, B., Danilova, L., . . . Papadopoulos, N. (2018). Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science, 359*(6378), 926–930. https://doi.org/10.1126/science.aar3247

Denny, P., Hagen, F. K., Hardt, M., Liao, L., Yan, W., Arellanno, M., . . . Fisher, S. J. (2008). The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *Journal of Proteome Research, 7*, 1994–2006. https://doi.org/10.1021/pr700764j

Diaz, L. A., & Bardelli, A. (2014). Liquid biopsies: Genotyping circulating tumor DNA. *Journal of Clinical Oncology, 32*, 579–586. https://doi.org/10.1200/JCO.2012.45.2011

Dowling, P., Wormald, R., Meleady, P., Henry, M., Curran, A., & Clynes, M. (2008). Analysis of the saliva proteome from patients with head and neck squamous cell carcinoma reveals differences in abundance levels of proteins associated with tumour progression and metastasis. *Journal of Proteomics, 71*, 168–175. https://doi.org/10.1016/j.jprot.2008.04.004

Du, J., & Zhang, L. (2017). Analysis of salivary microRNA expression profiles and identification of novel biomarkers in esophageal cancer. *Oncology Letters, 14*, 1387–1394. https://doi.org/10.3892/ol.2017.6328

Duffy, M. J., Shering, S., Sherry, F., McDermott, E., & O'Higgins, N. (2000). CA 15-3: A prognostic marker in breast cancer. *The International Journal of Biological Markers, 15*, 330–333.

Elashoff, D., Zhou, H., Reiss, J., Wang, J., Xiao, H., Henson, B., . . . Wong, D. T. (2012). Prevalidation of salivary biomarkers for oral cancer detection. *Cancer Epidemiology, Biomarkers & Prevention, 21*, 664–672. https://doi.org/10.1158/1055-9965.EPI-11-1093

Füzéry, A. K., Levin, J., Chan, M. M., & Chan, D. W. (2013). Translation of proteomic biomarkers into FDA approved cancer diagnostics: Issues and challenges. *Clinical Proteomics, 10*, 13. https://doi.org/10.1186/1559-0275-10-13

Gallo, A., Tandon, M., Alevizos, I., & Illei, G. G. (2012). The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PLoS One, 7*, e30679. https://doi.org/10.1371/journal.pone.0030679

García-Olmo, D. C., Domínguez, C., García-Arranz, M., Anker, P., Stroun, M., García-Verdugo, J. M., & García-Olmo, D. (2010). Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. *Cancer Research, 70*, 560–567. https://doi.org/10.1158/0008-5472.CAN-09-3513

Grassl, N., Kulak, N. A., Pichler, G., Geyer, P. E., Jung, J., Schubert, S., . . . Mann, M. (2016). Ultra-deep and quantitative saliva proteome reveals dynamics of the oral microbiome. *Genome Medicine, 8*, 44. https://doi.org/10.1186/s13073-016-0293-0

Gröschl, M. (2008). Current status of salivary hormone analysis. *Clinical Chemistry, 54*, 1759–1769. https://doi.org/10.1373/clinchem.2008.108910

Ha, M., & Kim, V. N. (2014). Regulation of microRNA biogenesis. *Nature Reviews. Molecular Cell Biology, 15*, 509–524. https://doi.org/10.1038/nrm3838

Han, Y.-N., Li, Y., Xia, S.-Q., Zhang, Y. Y., Zheng, J. H., & Li, W. (2017). PIWI proteins and PIWI-interacting RNA: Emerging roles in cancer. *Cellular Physiology and Biochemistry, 44*, 1–20. https://doi.org/10.1159/000484541

Han, X., Wang, J., & Sun, Y. (2017). Circulating tumor DNA as biomarkers for cancer detection. *Genomics, Proteomics & Bioinformatics, 15*, 59–72. https://doi.org/10.1016/j.gpb.2016.12.004

Han, N., Lu, H., Zhang, Z., Ruan, M., Yang, W., & Zhang, C. (2018). Comprehensive and in-depth analysis of microRNA and mRNA expression profile in salivary adenoid cystic carcinoma. *Gene, 678*, 349–360. https://doi.org/10.1016/j.gene.2018.08.023

He, M., & Zeng, Y. (2016). Microfluidic exosome analysis toward liquid biopsy for cancer. *Journal of Laboratory Automation, 21*, 599–608. https://doi.org/10.1177/2211068216651035

Helmerhorst, E. J., & Oppenheim, F. G. (2007). Saliva: A dynamic proteome. *Journal of Dental Research, 86*, 680–693. https://doi.org/10.1177/154405910708600802

Henson, B. S., & Wong, D. T. (2010). Collection, storage, and processing of saliva samples for downstream molecular applications. *Methods in Molecular Biology (Clifton NJ), 666*, 21–30. https://doi.org/10.1007/978-1-60761-820-1_2

Hsu, C. M., Lin, P. M., Wang, Y. M., Chen, Z. J., Lin, S. F., & Yang, M. Y. (2012). Circulating miRNA is a novel marker for head and neck squamous cell carcinoma. *Tumor Biology, 33*(6), 1933–1942.

Hu, S., Arellano, M., Boontheung, P., Wang, J., Zhou, H., Jiang, J., … Wong, D. T. (2008). Salivary proteomics for oral cancer biomarker discovery. *Cancer Research, 14*, 6246–6252. https://doi.org/10.1158/1078-0432.CCR-07-5037

Hui, A. B. Y., Lenarduzzi, M., Krushel, T., Waldron, L., Pintilie, M., Shi, W., … Liu, F. F. (2010). Comprehensive MicroRNA profiling for head and neck squamous cell carcinomas. *Cancer Research, 16*, 1129–1139. https://doi.org/10.1158/1078-0432.CCR-09-2166

Ilié, M., & Hofman, P. (2016). Pros: Can tissue biopsy be replaced by liquid biopsy? *Translational Lung Cancer Research, 5*, 420–423. https://doi.org/10.21037/tlcr.2016.08.06

Inal, C., Yilmaz, E., Piperdi, B., Perez-Soler, R., & Cheng, H. (2015). Emerging treatment for advanced lung cancer with EGFR mutation. *Expert Opinion on Emerging Drugs, 20*, 597–612. https://doi.org/10.1517/14728214.2015.1058778

Jahr, S., Hentze, H., Englisch, S., Hardt, D., Fackelmayer, F. O., Hesch, R. D., & Knippers, R. (2001). DNA fragments in the blood plasma of cancer patients: Quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Research, 61*, 1659–1665.

Kalra, H., Simpson, R. J., Ji, H., Aikawa, E., Altevogt, P., Askenase, P., … Mathivanan, S. (2012). Vesiclepedia: A compendium for extracellular vesicles with continuous community annotation. *PLoS Biology, 10*, e1001450. https://doi.org/10.1371/journal.pbio.1001450

Katsiougiannis, S., Chia, D., Kim, Y., Singh, R. P., & Wong, D. T. (2017). Saliva exosomes from pancreatic tumor–bearing mice modulate NK cell phenotype and antitumor cytotoxicity. *The FASEB Journal, 31*, 998–1010. https://doi.org/10.1096/fj.201600984R

Kawahara, R., Bollinger, J. G., Rivera, C., Ribeiro, A. C., Brandão, T. B., Paes Leme, A. F., & MJ, M. C. (2016). A targeted proteomic strategy for the measurement of oral cancer candidate biomarkers in human saliva. *Proteomics, 16*, 159–173. https://doi.org/10.1002/pmic.201500224

Lau, C., Kim, Y., Chia, D., Spielmann, N., Eibl, G., Elashoff, D., … Wong, D. T. (2013). Role of pancreatic cancer-derived exosomes in salivary biomarker development. *The Journal of Biological Chemistry, 288*, 26888–26897. https://doi.org/10.1074/jbc.M113.452458

Lee, Y.-H., Kim, J. H., Zhou, H., Kim, B. W., & Wong, D. T. (2012). Salivary transcriptomic biomarkers for detection of ovarian cancer: For serous papillary adenocarcinoma. *Journal of Molecular Medicine, 90*, 427–434. https://doi.org/10.1007/s00109-011-0829-0

Li, Y., St. John, M. A. R., Zhou, X., Kim, Y., Sinha, U., Jordan, R. C., … Wong, D. T. (2004). Salivary transcriptome diagnostics for oral cancer detection. *Cancer Research, 10*, 8442–8450. https://doi.org/10.1158/1078-0432.CCR-04-1167

Li, Y., Zhou, X., St. John, M. A. R., & Wong, D. T. W. (2004). RNA profiling of cell-free saliva using microarray technology. *Journal of Dental Research, 83*, 199–203. https://doi.org/10.1177/154405910408300303

Lin, S., & Gregory, R. I. (2015). MicroRNA biogenesis pathways in cancer. *Nature Reviews. Cancer, 15*, 321–333. https://doi.org/10.1038/nrc3932

Lin, S. C., Liu, C. J., Lin, J. A., Chiang, W. F., Hung, P. S., & Chang, K. W. (2010). miR-24 up-regulation in oral carcinoma: Positive association from clinical and in vitro analysis. *Oral Oncology, 46*(3), 204–208.

Liu, C. J., Kao, S. Y., Tu, H. F., Tsai, M. M., Chang, K. W., & Lin, S. C. (2010). Increase of microRNA miR-31 level in plasma could be a potential marker of oral cancer. *Oral Diseases, 16* (4), 360–364.

Liu, X., Yu, H., Qiao, Y., Yang, J., Shu, J., Zhang, J., . . . Li, Z. (2018). Salivary glycopatterns as potential biomarkers for screening of early-stage breast cancer. *eBioMedicine, 28*, 70–79. https://doi.org/10.1016/j.ebiom.2018.01.026

Loo, J. A., Yan, W., Ramachandran, P., & Wong, D. T. (2010). Comparative human salivary and plasma proteomes. *Journal of Dental Research, 89*, 1016–1023. https://doi.org/10.1177/0022034510380414

Looi, M.-L., Zakaria, H., Osman, J., & Jamal, R. (2012). Quantity and quality assessment of DNA extracted from saliva and blood. *Clinical Laboratory, 58*, 307–312.

Matse, J. H., Yoshizawa, J., Wang, X., Elashoff, D., Bolscher, J. G., Veerman, E. C., . . . Wong, D. T. (2013). Discovery and prevalidation of salivary extracellular microRNA biomarkers panel for the noninvasive detection of benign and malignant parotid gland tumors. *Clinical Cancer Research, 19*, 3032–3038. https://doi.org/10.1158/1078-0432.CCR-12-3505

National Cancer Institute. (2011). NCI dictionary of cancer terms. *Journal of National Cancer Institute*. Accessed November 25, 2018, from https://www.cancer.gov/publications/dictionaries/cancer-terms

Navarro, M. A., Mesía, R., Díez-Gibert, O., Rueda, A., Ojeda, B., & Alonso, M. C. (1997). Epidermal growth factor in plasma and saliva of patients with active breast cancer and breast cancer patients in follow-up compared with healthy women. *Breast Cancer Research and Treatment, 42*, 83–86. https://doi.org/10.1023/A:1005755928831

Newman, A. M., Bratman, S. V., To, J., Wynne, J. F., Eclov, N. C., Modlin, L. A., . . . Diehn, M. (2014). An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nature Medicine, 20*, 548–554. https://doi.org/10.1038/nm.3519

Niu, Z., Pang, R. T. K., Liu, W., Cheng, R., & WSB, Y. (2017). Polymer-based precipitation preserves biological activities of extracellular vesicles from an endometrial cell line. *PLoS One, 12*, e0186534. https://doi.org/10.1371/journal.pone.0186534

Nonaka, T., & Wong, D. T. W. (2017). Saliva-exosomics in cancer: Molecular characterization of cancer-derived exosomes in saliva. *The Enzymes, 42*, 125–151. https://doi.org/10.1016/bs.enz.2017.08.002

Nonaka, T., & Wong, D. T. W. (2018). Liquid biopsy in head and neck cancer: Promises and challenges. *Journal of Dental Research, 97*, 701–708. https://doi.org/10.1177/0022034518762071

Ogawa, Y., Kanai-Azuma, M., Akimoto, Y., Kawakami, H., & Yanoshita, R. (2008). Exosome-like vesicles with dipeptidyl peptidase IV in human saliva. *Biological & Pharmaceutical Bulletin, 31*, 1059–1062.

Ohshiro, K., Rosenthal, D. I., Koomen, J. M., Streckfus, C. F., Chambers, M., Kobayashi, R., & El-Naggar, A. K. (2007). Pre-analytic saliva processing affect proteomic results and biomarker screening of head and neck squamous carcinoma. *International Journal of Oncology, 30*, 743–749.

Park, N. J., Li, Y., Yu, T., Brinkman, B. M., & Wong, D. T. (2006). Characterization of RNA in saliva. *Clinical Chemistry, 52*, 988–994. https://doi.org/10.1373/clinchem.2005.063206

Park, N. J., Zhou, H., Elashoff, D., Henson, B. S., Kastratovic, D. A., Abemayor, E., & Wong, D. T. (2009). Salivary microRNA: Discovery, characterization, and clinical utility for oral cancer detection. *Cancer Research, 15*, 5473–5477. https://doi.org/10.1158/1078-0432.CCR-09-0736

Park, N. J., Zhou, X., Yu, T., Brinkman, B. M. N., Zimmerman, B., Palaniswamy, V., & Wong, D. T. (2007). Characterization of salivary RNA by cDNA library analysis. *Archives of Oral Biology, 52*, 30–35. https://doi.org/10.1016/j.archoralbio.2006.08.014

Pepe, M. S., Etzioni, R., Feng, Z., Potter, J. D., Thompson, M. L., Thornquist, M., … Yasui, Y. (2001). Phases of biomarker development for early detection of cancer. *Journal of the National Cancer Institute, 93*, 1054–1061.

Phallen, J., Sausen, M., Adleff, V., Leal, A., Hruban, C., White, J., … Velculescu, V. E. (2017). Direct detection of early-stage cancers using circulating tumor DNA. *Science Translational Medicine, 9*, eaan2415. https://doi.org/10.1126/scitranslmed.aan2415

Pu, D., Liang, H., Wei, F., Akin, D., Feng, Z., Yan, Q., … Zhou, Q. (2016). Evaluation of a novel saliva-based epidermal growth factor receptor mutation detection for lung cancer: A pilot study. *Thoracic Cancer, 7*, 428–436. https://doi.org/10.1111/1759-7714.12350

Rai, B., Kaur, J., Jacobs, R., & Anand, S. C. (2011). Adenosine deaminase in saliva as a diagnostic marker of squamous cell carcinoma of tongue. *Clinical Oral Investigations, 15*, 347–349. https://doi.org/10.1007/s00784-010-0404-z

Schulz, B. L., Cooper-White, J., & Punyadeera, C. K. (2013). Saliva proteome research: Current status and future outlook. *Critical Reviews in Biotechnology, 33*, 246–259. https://doi.org/10.3109/07388551.2012.687361

Sharma, S., Gillespie, B. M., Palanisamy, V., & Gimzewski, J. K. (2011). Quantitative nanostructural and single-molecule force spectroscopy biomolecular analysis of human-saliva-derived exosomes. *Langmuir, 27*(23), 14394–14400.

Sharma, P., Ludwig, S., Muller, L., Hong, C. S., Kirkwood, J. M., Ferrone, S., & Whiteside, T. L. (2018). Immunoaffinity-based isolation of melanoma cell-derived exosomes from plasma of patients with melanoma. *Journal of Extracellular Vesicles, 7*, 1435138. https://doi.org/10.1080/20013078.2018.1435138

Siegel, R. L., Miller, K. D., & Jemal, A. (2018). Cancer statistics, 2018. *CA: A Cancer Journal for Clinicians, 68*, 7–30. https://doi.org/10.3322/caac.21442

Spielmann, N., & Wong, D. T. (2011). Saliva: Diagnostics and therapeutic perspectives. *Oral Diseases, 17*, 345–354. https://doi.org/10.1111/j.1601-0825.2010.01773.x

Streckfus, C., Bigler, L., Dellinger, T., Dai, X., Kingman, A., & Thigpen, J. T. (2000). The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: A preliminary study. *Cancer Research, 6*, 2363–2370.

Streckfus, C., Bigler, L., Tucci, M., & Thigpen, J. T. (2000). A preliminary study of CA15-3, c-erbB-2, epidermal growth factor receptor, cathepsin-D, and p53 in saliva among women with breast carcinoma. *Cancer Investigation, 18*(2), 101–109.

Stroun, M., Anker, P., Maurice, P., & Gahan, P. B. (1977). Circulating nucleic acids in higher organisms. *International Review of Cytology, 51*, 1–48.

Stroun, M., Anker, P., Maurice, P., Lyautey, J., Lederrey, C., & Beljanski, M. (1989). Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology, 46*, 318–322. https://doi.org/10.1159/000226740

Summerer, I., Unger, K., Braselmann, H., Schuettrumpf, L., Maihoefer, C., Baumeister, P., … Zitzelsberger, H. (2015). Circulating microRNAs as prognostic therapy biomarkers in head and neck cancer patients. *British Journal of Cancer, 113*, 76–82. https://doi.org/10.1038/bjc.2015.111

Sun, Y., Huo, C., Qiao, Z., Shang, Z., Uzzaman, A., Liu, S., … Xiao, H. (2018). Comparative proteomic analysis of exosomes and microvesicles in human saliva for lung cancer. *Journal of Proteome Research, 17*, 1101–1107. https://doi.org/10.1021/acs.jproteome.7b00770

Tajmul, M., Parween, F., Singh, L., Mathur, S. R., Sharma, J. B., Kumar, S., … Yadav, S. (2018). Identification and validation of salivary proteomic signatures for non-invasive detection of ovarian cancer. *International Journal of Biological Macromolecules, 108*, 503–514. https://doi.org/10.1016/j.ijbiomac.2017.12.014

Théry, C., Amigorena, S., Raposo, G., & Clayton, A. (2006). Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Current Protocols in Cell Biology, Chapter 3*, Unit 3.22. https://doi.org/10.1002/0471143030.cb0322s30

Thierry, A. R., El Messaoudi, S., Gahan, P. B., Anker, P., & Stroun, M. (2016). Origins, structures, and functions of circulating DNA in oncology. *Cancer Metastasis Reviews, 35*, 347–376. https://doi.org/10.1007/s10555-016-9629-x

Wang, Y., Springer, S., Mulvey, C. L., Silliman, N., Schaefer, J., Sausen, M., & Agrawal, N. (2015). Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. *Science Translational Medicine, 7*, 293ra104. https://doi.org/10.1126/scitranslmed.aaa8507

Wang, S., Tsui, S. T., Liu, C., Song, Y., & Liu, D. (2016). EGFR C797S mutation mediates resistance to third-generation inhibitors in T790M-positive non-small cell lung cancer. *Journal of Hematology and Oncology, 9*, 59. https://doi.org/10.1186/s13045-016-0290-1

Wei, F., Lin, C.-C., Joon, A., Feng, Z., Troche, G., Lira, M. E., … Wong, D. T. (2014). Noninvasive saliva-based EGFR gene mutation detection in patients with lung cancer. *American Journal of Respiratory and Critical Care Medicine, 190*, 1117–1126. https://doi.org/10.1164/rccm.201406-1003OC

Wei, F., Yang, J., & Wong, D. T. W. (2013). Detection of exosomal biomarker by electric field-induced release and measurement (EFIRM). *Biosensors & Bioelectronics, 44*, 115–121. https://doi.org/10.1016/j.bios.2012.12.046

Wood, N., & Streckfus, C. F. (2015). The expression of lung resistance protein in saliva: A novel prognostic indicator protein for carcinoma of the breast. *Cancer Investigation, 33*, 510–515. https://doi.org/10.3109/07357907.2015.1081920

Wu, M., Ouyang, Y., Wang, Z., Zhang, R., Huang, P. H., Chen, C., … Huang, T. J. (2017). Isolation of exosomes from whole blood by integrating acoustics and microfluidics. *Proceedings of the National Academy of Sciences of the United States of America, 114*, 10584–10589. https://doi.org/10.1073/pnas.1709210114

Wu, Z.-Z., Wang, J.-G., & Zhang, X.-L. (2009). Diagnostic model of saliva protein finger print analysis of patients with gastric cancer. *World Journal of Gastroenterology, 15*, 865–870. https://doi.org/10.3748/wjg.15.865

Xiao, H., & Wong, D. T. W. (2012). Method development for proteome stabilization in human saliva. *Analytica Chimica Acta, 722*, 63–69. https://doi.org/10.1016/j.aca.2012.02.017

Xiao, H., Zhang, Y., Kim, Y., Kim, S., Kim, J. J., Kim, K. M., … Wong, D. T. (2016). Differential proteomic analysis of human saliva using tandem mass tags quantification for gastric cancer detection. *Scientific Reports, 6*, 22165. https://doi.org/10.1038/srep22165

Xiao, H., Zhang, L., Zhou, H., Lee, J. M., Garon, E. B., & Wong, D. T. (2012). Proteomic analysis of human saliva from lung cancer patients using two-dimensional difference gel electrophoresis and mass spectrometry. *Molecular & Cellular Proteomics, 11*, M111.012112. https://doi.org/10.1074/mcp.M111.012112

Yan, W., Apweiler, R., Balgley, B. M., Boontheung, P., Bundy, J. L., Cargile, B. J., … Wong, D. T. (2009). Systematic comparison of the human saliva and plasma proteomes. *Proteomics. Clinical Applications, 3*, 116–134. https://doi.org/10.1002/prca.200800140

Yang, J., Wei, F., Schafer, C., & Wong, D. T. W. (2014). Detection of tumor cell-specific mRNA and protein in exosome-like microvesicles from blood and saliva. *PLoS One, 9*, e110641. https://doi.org/10.1371/journal.pone.0110641

Zhang, L., Farrell, J. J., Zhou, H., Elashoff, D., Akin, D., Park, N. H., … Wong, D. T. (2010). Salivary transcriptomic biomarkers for detection of resectable pancreatic cancer. *Gastroenterology, 138*, 949–957.e1–7. https://doi.org/10.1053/j.gastro.2009.11.010

Zhang, J., Li, S., Li, L., Li, M., Guo, C., Yao, J., & Mi, S. (2015). Exosome and Exosomal MicroRNA: Trafficking, sorting, and function. *Genomics Proteomics Bioinformatics, 13*, 17–24. https://doi.org/10.1016/j.gpb.2015.02.001

Zhang, L., Xiao, H., Karlan, S., Zhou, H., Gross, J., Elashoff, D., … Wong, D. T. (2010). Discovery and preclinical validation of salivary transcriptomic and proteomic biomarkers for the non-invasive detection of breast cancer. *PLoS One, 5*, e15573. https://doi.org/10.1371/journal.pone.0015573