Chapter

Diagnostic Potential of Salivary Exosomes in Oral Cancer

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Abstract

"Omics" based concepts and techniques are gaining momentum in the field of oral medicine, spurred on by rapid advancements within the field of precision diagnostics and therapeutics. Oral cancer, specifically oral squamous cell carcinoma is the most common head and neck cancer, posing both diagnostic and prognostic challenges globally. Saliva offers several advantages as a diagnostic tool and has gained recognition as a biological medium for liquid biopsy. Salivary biomarkers, such as exosomes not only contain the full spectrum of genomic, lipidomic and proteomic material from its cell of origin, but are also more stable and consistently measurable in saliva due to their phospholipid structural protection of their merchandise/contents. Salivary exosomes are mediators in communication and transfer of contents between cancer and normal cells and thus key role players in mediating the tumor environment. Even though exosomes have been widely employed to investigate systemic diseases including head and neck cancers, unraveling the biologic mechanisms, scope of application of salivary tumor-derived exosomes and overcoming restrictions in this emergent field of saliva-exosomics warrants further investigation.

Keywords: Saliva, exosomes, oral cancer, exosomics, omics-based approaches

1. Introduction

Despite the fact, that there is a relatively wider coverage and application of several emerging molecular and omics-based techniques, in the field of medicine; many of these concepts (and techniques) are only beginning to gain recognition in the field of dentistry [1]. The completion of the human genome project [2–4], has expanded the trajectory for precision diagnostic and therapeutic potential thereof across many biomedical fields [5, 6]. The clinical lexicon in the post-genomic era is now burgeoning with various personalized application of genomics (and phenomics) to improving the cancer management pipelines [1, 6–9].

Orthogonal (but complementary) multimodal approaches to conventional histopathology [10, 11], such as liquid biopsies technologies have emerged as useful tools for clinical oncology [11–13], early tumor diagnosis and biomonitoring [14–17], as well as therapeutic decision making and delivery [18]. Nano-scaled multivesicular exosomes have emerged as important components of the tumor circulome that has significantly improved the cancer diagnostic field [11, 14]. Furthermore, salivary exosomes have been applied to improve the diagnosis of various cancers [19–26],

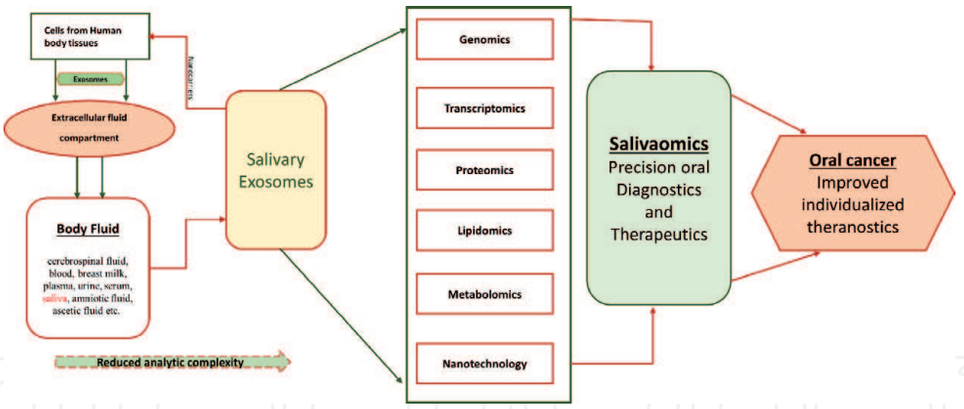

Figure 1.
Overview of origin and theranostic value of exosomes in oral cancer.

including oral cancers [25, 26]. The focus of this chapter is to review the applications and prospects of salivary exosomes in oral cancer detection (**Figure 1**).

2. Salivary diagnostics

Human saliva is a multifunctional biological fluid, which facilitates digestion, swallowing, tasting, tissue lubrication and protection against infectious organisms. It is comprised largely of water (99%) and other biochemical substances such as proteins, nucleic acids, mucins, immunoglobulins, a variety of electrolytes and lipids [27, 28]. Whole saliva production is derived from all the salivary glands including the gingival crevicular fluid [29]. Saliva, which has been used for diagnostic purposes for over 2000 years, plays a key role in the maintenance of general and particularly oral health homeostasis [30]. The health of individuals has been determined by salivary changes such as amount produced, smell, ropiness and gustatory sensation [31, 32].

Components of the salivary glands are responsible for production, modification, transportation and secretion of saliva into the oral cavity (via acinar cells, various ductal system cells, and myoepithelial cells) [33]. The close proximity of a network of highly permeable blood capillaries to the saliva producing acinar cells facilitates the free exchange of blood-derived molecules into the acinar cells [34], which enter the salivary tissues either via transcellular (passive and active transport) or paracellular (extracellular ultrafiltration) routes [35, 36]. This transfer potentially influences the molecular constituency of oral fluids. Diffusion is the most common transport mechanism of molecules from blood into the saliva and this process is driven/influenced by the size and the electric charge of the molecules [37]. Active transport involves the transcellular transportation of blood into saliva via secretory acinar cells of the salivary glands. Ultra-filtration is the major mode by which molecules are transported into saliva via the paracellular route, whereby small sized blood molecules filter into saliva through the spaces between ductal and acinar cells [37]. Salivary acini secrete saliva into collecting ducts, where sodium reabsorption, and bicarbonate and potassium are secretion takes place, thus altering the composition of the saliva [38, 39]. Even though blood molecules transported through ultra-filtration into saliva are usually in low concentration in comparison to their levels in blood, this mode of transport enables blood molecules such as DNA, RNA, proteins, metabolites and exosomes into saliva through the salivary gland. This confers the possibility for oral fluids to harbor molecular information indicative of an individual's current state of health. This information may be reflected by changes in the concentrations of these molecules or mutation in the genetic constitution of the

molecules which are also present in saliva - serving as potential salivary biomarkers for diagnosis, prognosis and monitoring of therapeutic responses [40].

Many body fluids have been explored as alternative sources for biomarkers in molecular diagnosis of cancers, genetic, immunological and other systemic diseases [41–45]. However, blood, urine and saliva are the most used media for discovery of biomarkers [46]. Saliva is a rich source of proteins and its DNA, RNA, and protein content is analogous to that of blood with significant commonality in hormones, antibodies and other molecules. Most of the resident salivary protein constituents are synthesized within the salivary glands, with the rest transported from blood or lymph into saliva and used as biomarkers for disease diagnosis and screening purposes [47].

Saliva has the advantages over blood in that it is a readily available specimen which can be collected by non-invasive techniques and is recommended as the diagnostic medium in vulnerable populations such as children. The retrieval of multiple salivary samples from the same individual is possible with minimal discomfort and saliva is safer to work with when compared with blood samples. For example, there are some factors in saliva which help to inhibit HIV infectivity thereby limiting the rate of HIV transmission through the oral cavity [48]. Saliva sample processing is more economical and does not clot making it easier to store and ship with less manipulation. However, the low level of protein detection in saliva is sensitive to the method of saliva collection and specimen contamination. Normal high-abundance salivary constituents such as amylase and proline rich proteins (during stimulated salivary collection methods especially), may dilute the presence of low-level proteins, which may be more important biomarkers.

Salivary biomarkers are miniscule and measured in (picograms), detection of which can only be achieved by techniques which are both sensitive and specific enough to discriminate between them [49]. Technological advances in diagnostic detection methods (next generation sequencing, mass spectrometry, genome wide association studies and other screening techniques) have paralleled the demand for improved diagnostic test accuracy of salivary genomic and proteomic biomarkers, thereby conferring distinct advantages for saliva in the diagnosis and monitoring of diseases such as oral cancer and precancer.

Salivary exosomes, which are nano-sized salivary biomarkers equipped with all the molecular cargo from the parent cells, have become increasingly detectable due to their stability in the circulation and bodily fluids. They have been extensively explored as diagnostic tools for local and systemic diseases [50].

2.1 Salivary exosome physiology

Fusion of nano-sized (30–100 nm in diameter) multivesicular bodies (MVB) [51] derived from the endocytic pathway with plasma membrane was discovered over 30 years ago [52]. Johnstone et al., detected the release of small vesicles into extracellular spaces by reticulocyte MVB's and observed that their enzymatic activity mirrored that of the cell culture from which they were shed [52]. These bodies (exosomes), originally believed to be involved in waste disposal, due to their resistance to degradation by lysozymes, is now more understood to subserve vital biological functions when released as extracellular vesicles [53]. These functions are influenced/determined by their target cell with which they interact and include cellular communication and homeostasis, immune control (they contain IgA), RNA processing and transport of drugs [53–55].

Exosomal release (after formation of intraluminal vesicles), can be compared to the reversal of the endocytosis process, permits their evaluation in the extracellular body fluid environments [56]. The exocytotic release of exosomes into the extracellular domain, reveals that they naturally contain key molecular components derived

from the parent cell relating to membrane transport, lipid metabolism and extracellular matrix formation [21, 57]. In addition, cytoplasmic nucleic acid contents such as mRNA and microRNA have been found in exosomes [21, 58, 59]. Considering the important contents as highlighted here, exosomes have been identified to play crucial roles in cell-to-cell communication [21]. All exosomes regardless of their origin possess both shared conserved and cell-specific proteins. Emerging knowledge has associated exosomes with the development of physiological and pathological perturbations [60, 61]. For instance, cancer exosomes have been found to be capable of a range of tumor-promoting activities, such as immunomodulation, development of pre-metastatic niches, as well as dysregulation of angiogenesis [62–64]. Furthermore, cancer exosomes are vital indicators of potentially malignant events in the tumor microenvironment and may exhibit pheno-genomic perturbation biomarkers of cancer [65, 66].

2.2 Diagnostic benefits of exosomes in body fluids

Due to exosomal release into the extracellular compartment, they are abundantly found in most body fluids such as cerebrospinal fluids, blood, breast milk, plasma, urine, serum, saliva, amniotic fluid, semen and ascetic fluid [21, 52–55]. Exosomes are highly suitable substrates for biomarker signature discovery. Because of the content-protective packaging of their rich cargo (by lipid membranes) from extracellular lytic enzymes, and significantly lower complexity of its contents in comparison to whole tissue analysis [53].

Analyzing exosomal shuttle RNA (esRNA) in maternal blood, has been proposed as a potential surrogate prenatal diagnostic tool, to avoid risky invasive procedures such as chorionic villus sampling and amniocentesis [54]. This could potentially lower the risk of surgical injuries and miscarriages. Even though, cell free fetal DNA (cffDNA) has been previously used for the prenatal diagnostic purposes, the low content of fetal cffDNA has reduced the accuracy of this method [54, 56]. Information about cancer risk and genetic disease predisposition can be potentially gleaned from exosomal esRNA content analysis.

The use of novel liquid biopsy-based cancer diagnostic tools has significantly improved the precision and efficacy of individualized medicine, particularly in resource-limited settings [11]. Exosomes are capable of providing robust molecular tumor information about cells of origin, are retrievable from easily accessible body fluids; and hence are highly useful for early detection and follow-up of cancer [55].

2.3 Salivary exosomes and oral cancer

Exosomes have been successfully isolated from saliva [19–25]; and the presence of lipids, proteins and nucleic acids in exosomes, makes salivary exosomes attractive substrates for omics analysis (a.k.a Salivaomics) [57–60]. It has become emergent, that salivary constituents (e.g. mRNAs, proteins, miRNAs, microbes and metabolites such as lipids) may be detected in exosomes and be used as biomarkers for diseases (both local and distal) [54]. Structural, proteomics and transcriptomics analysis of salivary exosomes has been successfully carried out [61–65]; and salivary exosomes are fast becoming key tools in cancer biomarker theranostics. Exosomes have been revealed as valuable indicators of the micro-environments and perpetrators of cancer intercellular communication [25].

The mean diameter and protein content are used as the basis upon which exosomes isolated from saliva are structurally subdivided into two types (I and II which are ca. 85 nm and 40 nm in diameter, respectively) [20]. Since epithelial barriers between blood vessels and salivary gland structures can be crossed by exosomes [66, 67], they have become important tools for essential transport of key signatures

between blood and saliva (which is believed to be an ultrafiltrate of blood) [67–69]. Important molecular information may be exchanged via transudation, ultrafiltration or selective transport based on their size or presence of transporter molecules [67].

Of all the head and neck cancers, oral squamous cell carcinoma is the most prevalent, often diagnosed in advanced stage and is associated with a low survival and poor prognosis. Early detection of oral cancer is a key goal in epidemiological cancer control and successful management thereof. Using salivary exosomes for identification of oral cancer biomarkers is potentially a highly sensitive, cost-effective and non-invasive point-of-care technique for detecting oral cancer that may be subclinical or missed by routine histological diagnostic approaches [70]. Evaluation of these vesicles shed by cancer cells via multivesicular bodies (MVB's) into saliva is a viable approach for biomarker detection in oral cancer (**Figure 2**). Salivary exosomes have played key roles in diagnosis of systemic diseases [51] and have been key player in the molecular characterization of cancer [24]. Due to its reduced complexity as compared to other body fluids, exosomes are reliable tools for early diagnosis of oral cancer and its use as a diagnostic tool may significantly improve cancer survival rates [24].

Due to its nano-scaled structure, human salivary exosomes have been identified as potential carriers for non-invasive delivery of cancer biomarkers [22]. For example, electrochemical sensing methods, such as electric field-induced release and measurement (EFIRM) approach, has been used to improve the field of salivary liquid biopsy [23]. Not least, salivary exosomes have been used to enhance the detection of human papilloma virus (HPV) positive oropharyngeal cancers [26].

The critical role of tumor-derived exosome in cancer in largely due to the presence of tumor-specific signatures within its functional cargos, which includes proteins, miRNA and mRNA (**Table 1**).

The significant physiological interaction and overlap of the blood and salivary proteome (ca. 20–30%) makes exosomal protein biomarkers attractive and many cancer-related exosomal proteins have been identified from oral cancer [23, 82, 83]. Potential proteomic biomarkers of oral cancer such as CFB, CD59, A1BG, M2b, CAT, MRP14, PFN, M2BP, ADA, S100, CFL1, IGHG, TF, IL-1B and IL-8S have been

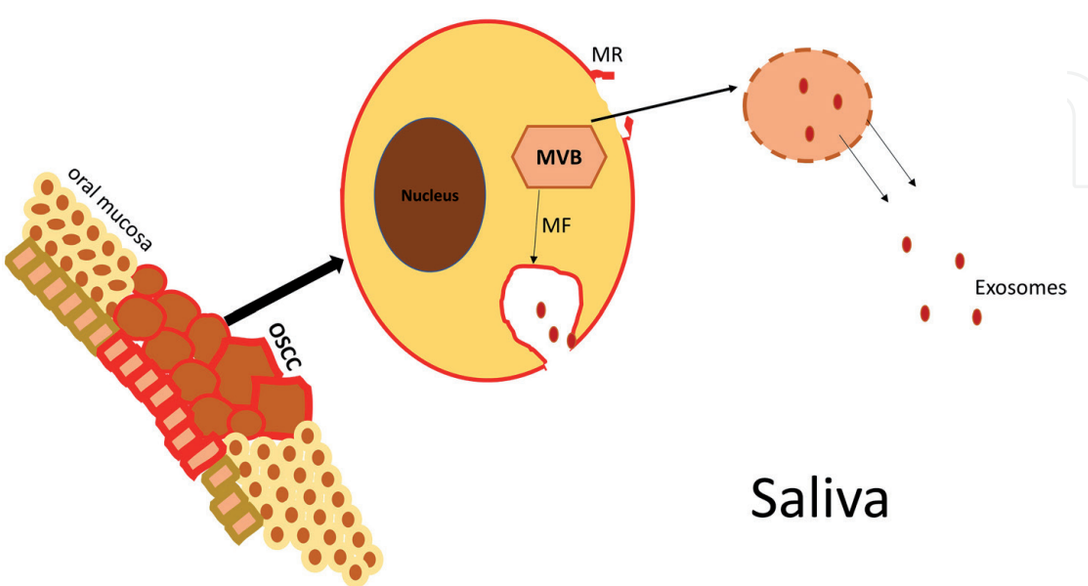

Figure 2.
Pathways for escape of exosomes into saliva. Oral squamous carcinoma cells (OSCC) may release exosomes into saliva, either by fusion of the multivesicular body (MVB) with the plasma membrane (MF) or by plasma membrane rupture (MR) and direct release through endosomal membrane.

Biomarker	Type	Sample	Methods	References
A2M, HPA, MUC5B, LGALS3BP, IGHA1, PIP, PKM1/M2, GAPDH	Protein	saliva	Mass spectrometry analysis and proteomics data analysis	Winck et al. [71]
miRNA-21	miRNA	OSCC cell line	miRNA sequencing	Li et al. [72]
miR-1246	miRNA	OSCC cell line	MicroRNA microarray	Sakha et al. [73]
miR-200c-3p	miRNA	OSCC cell line	integrated microarray	Kawakubo-Yasukochi et al. [74]
miR-34a-5p	miRNA	OSCC cell line	miRNA sequencing	Li et al. [75]
PF4V1, CXCL7, F13A1, and ApoA1	protein	serum	RT-PCR	Li et al. [76]
miR-101-3p	miRNA	OSCC cell line	Microarray analysis	Xie et al. [77]
miR-382-5p	miRNA	OSCC cell line	RT-PCR	Sun et al. [78]
miR-24-3p	miRNA	saliva	RT-PCR	He et al. [79]
miR-21-5p	miRNA	OSCC cell line	RT-PCR	Chen et al. [80]
miR-155	miRNA	OSCC cell line	RT-PCR.	Kirave et al. [81]

Table 1.
Exosome biomarker for oral cancers.

identified from whole saliva [84–88]. However, protein biomarkers such as MUC5B, A2M, LGALS3BP, HPa, GAPDH, IGHA1, PIP and PKM1/M2 have been specifically identified to be exosomal protein biomarkers of oral cancer with a classification accuracy of 90% [80, 89]. Zlotogorski-Hurvitz et al. (2016), identified CD9/−81 downregulation and CD 63 upregulation in exosomes as early diagnostic protein biomarkers of oral cancer [90]. Furthermore, salivary exosomes isolated from HPV-positive oropharyngeal cancer cell lines has been found to underexpress cyclin D1 and p53 and overexpress p16, T-cell inhibitory protein PTPN11 and E6/E7 proteins [91].

Via their interaction with mRNA, micro RNA's (miRNA) are involved in a number of physiological and disease processes (when there is aberrant expression). MiRNAs are small non-coding RNAs that mediate destabilization and/or translational repression of target messenger RNA (mRNA) molecules thereby reducing final protein output. Exosomes are a rich source of miRNA's, provides a vehicle for cell to cell transporting to alter cellular functions as well as offer protection in the extracellular environment. Exosomal miRNAs have been investigated as candidate screening tools (miR-24-3p) [92], in chemoresistance (miR-21) [81], regulating tumor progression (miR-34-5p) [93] and miR-342–3p and miR-1246 [93] and miR-382-5p [78].

In oral cancer, the intercellular transfer of molecules (such as miRNA's) by cancer associated fibroblast influences the tumor microenvironment. miR-21 represents one of the most abundant miRs transported within EV cargos secreted by oral cancer cells and is a well-established oncogenic miR whose major targets include the tumor suppressors. Its exosomal hideout contributes towards chemoresistance

due to the camouflage provided by its vehicle during intercellular transfer of the oncogenic miR. It is thus also an important chemotherapeutic precision target in cancers [81]. Li et al. [94], in a study of 108 patients with OSCC, observed that tumor exosomal miR 21 was upregulated in hypoxic cancer cells as well as internalized by normoxic cells. Exosomal miR-34-5p transfer between CAF's and neighboring OSCC cells played an important role in regulating tumor progression. Sakha et al. [73], demonstrated that effect that intercellular transfer of exosomal oncogenic miRNA's (miR-342–3p and miR-1246) could be delivered were evaluated for their role in could have on cancer development and progression by influencing cell motility and invasiveness [73]. Exosomal miR-382-5p in cancer-associated fibroblast (CAF) mediated OSSC migration and invasion by evaluation of tissue samples from 47 patients who had OSSC tumor resection. The results showed that CAF's transfer miR-382-5p associated with migration and invasion [78]. The expression of exosomal miR-24-3p was found to be higher in salivary exosomes from OSCC patients compared to healthy controls. The AUC for miR-24-3p was 0.738 and could significantly distinguish OSCC patients from normal individuals with 64.4% sensitivity and 80% specificity in 49 patients with OSSC.

The functional cargos which include mRNA has been considered as potential biomarker in the diagnosis and monitoring and treatment of cancer. Valadi et al. first described the presence of mRNAs in exosomes in 2007 [95]. Subsequently, studies have shown the transfer of bioactive mRNAs (tumor suppressor genes or oncogenes) from a malignant cell to a normal cell led to a change in the phenotype and malignant transformation of the normal cell [95–97].

Few studies have identified mRNA in saliva of oral cancer patients. Li et al. identified potential mRNA biomarker which include IL8, SAT, DUSP, IL1B, OAZ1, H3F3A and S100P, in saliva from oral cancer patients [98]. The study showed that the combination of these biomarkers was highly sensitive and specific in differentiating between OSSC and healthy [98]. An in vitro study showed that oral squamous cell carcinoma cell line (PCI-13, UMSCC47) triggered significant increase expression level of IGFBP-3 mRNA and VEGF mRNA in recipient cells [99].

Even though exosomes have been widely employed to investigate head and neck squamous cell carcinomas [100–103], unraveling the biologic mechanisms and application of salivary tumor -derived exosomes is still an evolving science [67]. This emergent field of saliva-exosomics warrants further investigation.

3. Conclusions

Salivary exosomes provide viable, consistent and stable sources of cancer biomarkers. The scope of its utility as well as understanding the molecular mechanisms which underpins it, requires further investigation. Future studies refining the methodology for extracellular vesicle isolation and cleansing presents the greatest challenge that is needed to overcome the restrictions to exploring the full scope of salivary exosomes in systemic diseases including oral cancer.

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Conflict of interest

The authors declare no conflict of interest.

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