Diagnostic biomarkers in head and neck cancer

Abstract

Head and neck cancer is the seventh most common cancer worldwide. It remains one of the leading causes of death, and its early detection is crucial. Liquid biopsy has emerged as a promising tool for detecting and monitoring the disease status of patients with early and advanced cancers. Circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and exosomal miRNAs have received enormous attention because of their apparent clinical implications. Analyses of these circulating biomarkers have paved the way for novel therapeutic approaches and precision medicine. A growing number of reports have implicated the use of circulating biomarkers for detection, treatment planning, response monitoring, and prognosis assessment. Although these new biomarkers can provide a wide range of possible clinical applications, no validated circulating biomarkers have yet been integrated into clinical practice for head and neck cancer. In this chapter, we summarize the current knowledge of circulating biomarkers in this field, focusing on their feasibility, limitations, and key areas of clinical applications. We also highlight recent advances in salivary diagnostics and their potential application in head and neck cancer.

1. Introduction

Despite advances in surgery and therapeutic strategies, the overall survival of head and neck cancer patients has remained unchanged for decades. Head and neck cancers represent the seventh most common cancer with estimated 880,000 new cases and 450,000 deaths worldwide in 2018 [1]. Most predominant histological type is squamous cell carcinoma (SCC) that mainly occurs in the oral cavity, oropharynx, hypopharynx, and larynx. Traditional cancer-screening techniques such as imaging and protein biomarkers are not sufficient for early detection.

In the era of personalized medical treatment, knowing specific cancer information is essential as it guides treatment decisions. Tissue biopsy is a standard method, but the limited sampling is often insufficient to capture the heterogeneity and evolution of tumors [2]. Liquid biopsy has been increasingly considered as an option for molecular characterization and detection of cancer as it can provide real-time information about cancer in a minimally invasive manner [3]. Circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and exosomal miRNAs are emerging biomarkers that can be applied to cancer detection, treatment planning, and response monitoring [4]. Notably, ctDNA and exosomal miRNAs have been shown to be present in multiple body fluids, including saliva, and are very promising biomarkers for cancer [5]. In this chapter, we summarize a current array of discovered circulating biomarkers (ctDNA, CTCs, and exosomal miRNAs) and their potential clinical applications in head and neck cancer. Early detection of cancer, particularly before metastatic spread, is crucial for early intervention and improving prognosis.

2. Circulating Tumor DNA and Circulating Tumor Cells

2.1. Early Detection

Circulating tumor DNA is a cancer-derived cell-free DNA in circulating blood. Blood from cancer patients had higher concentrations of circulating tumor DNA as compared to healthy individuals [6]. In 1994, Vasioukhin et al. and Sorenson et al. demonstrated the presence of tumor-specific mutations of the NRAS and KRAS genes in the plasma ctDNA [7, 8]. ctDNA mainly originates from apoptotic or necrotic tumor cells and contains the mutations present in the tumor (Fig. 1). Somatic mutations are tumor specific, and evaluation of these unique genetic changes offers the potential for better diagnostic accuracy. Several studies have demonstrated a high concordance of mutational profiles between plasma ctDNA and matched tumor samples in lung cancer [9], breast cancer [10, 11], and colorectal cancer [12, 13].

A recent proof-of-principle study reported ctDNA to be a biomarker in head and neck cancer [14]. In a cohort of 93 patients with HNSCC, including 20 cases of early stage cancer, plasma and saliva samples were screened for somatic mutations (TP53, PIK3CA, NOTCH1, FBXW7, CDKN2A, NRAS, and HRAS) and human papillomaviruses (HPV16 and 18) (Table 1). Plasma ctDNA was shown to be a more sensitive biomarker than salivary ctDNA for oropharynx, hypopharynx, and larynx cancer (plasma ctDNA: 86%–100% vs. salivary ctDNA: 47%–70%). However, salivary ctDNA showed better sensitivity than plasma ctDNA (100% vs. 80%) in oral cancer, indicating that oral cancer–derived DNA is more readily detected in saliva due to the close proximity of the tumor to saliva. Importantly, when both plasma and saliva were tested in combination, the overall ctDNA detection rate was 96%, irrespective of tumor location or stage. These findings demonstrate the importance of examining combination or appropriate bodily fluids according to tumor type to achieve the highest sensitivity. TP53 was the most frequently detected ctDNA in plasma of oral cancer patients (85%) and this was also the case for tumors of other anatomical sites (oropharynx 100%, hypopharynx 100%, larynx 86%). Despite high prevalence rates of TP53 ctDNA, HPV 16 DNA was not detected in oral cancer compared to 92% in oropharyngeal cancer. This difference may be due to the low number of HPV-positive oral cancer in this cohort because TP53 mutations and HPV-positive cancer are known to be mutually exclusive [15]. The Cancer Genome Atlas Network showed high TP53 mutation rate (86%) in 243 HPV-negative samples, while only 1 out of 36 HPV-positive cases (2.8%) had a non-synonymous TP53 mutation, which is consistent with this data [16].

For cancers with a viral etiology such as nasopharyngeal carcinoma, detection of the cancer-associated viral DNA may provide a good strategy for identifying individuals with early stage disease. Chan et al. screened asymptomatic volunteers for plasma Epstein-Barr virus (EBV) DNA and found 69 of the 1,318 participants (5.2%) had viral DNA, among whom 3 individuals were diagnosed with nasopharyngeal carcinoma [17]. They further confirmed this result in a prospective cohort with a total of 20,174 participants, demonstrating 34 of 309 participants (11%) who had persistently EBV-positive results on the repeated blood test developed nasopharyngeal carcinoma [18]. Population screening of virion DNA in plasma is a very promising approach to detect early-stage cancer.

Primary and metastatic tumors release subsets of CTCs into the blood (Fig. 1). An important hypothesis is that CTCs mirror tumor heterogeneity, and increased CTC levels exhibit diagnostic features. CTCs have been tested in numerous studies for diagnosis of primary tumors and metastatic relapse [19]. Nichols et al. and He et al. reported that CTCs were detected in 6 of 15 (40.0%) and 3 of 9 (33.3%) patients with head and neck cancer, respectively [20, 21]. Buglione et al. found that CTCs were more frequently found in advanced stages of head and neck cancer than in its early stages [22]. Moreover, Jatana et al. and Gröbe et al. reported that an increased number of CTCs was correlated with poorer prognosis, and the presence of CTCs was correlated with locoregional relapse [23, 24]. However, CTCs seem to be much less sensitive than ctDNA for early cancer detection. Bettegowda et al. found that no CTCs were detected in early stage bladder, breast, and colorectal cancers, whereas ctDNA was detected in 81% of these cancers [11]. These findings suggest that CTC is more likely to be a prognostic marker rather than an early diagnostic marker in cancer.

2.2. Treatment Selection

CTCs can be exploited as surrogate biopsy specimens to investigate the presence of drug targets. Cetuximab, a monoclonal antibody targeting the extracellular domain of the epidermal growth factor receptor (EGFR), is approved for the treatment of advanced HNSCC in combination with radiotherapy and chemotherapy [25, 26]. Measuring cell surface expression of EGFR on CTCs provides critical information for planning anti-EGFR treatment. In support of this, cetuximab treatment was more effective in reducing EGFR-positive CTCs than conventional chemotherapy in HNSCC [27].

EGFR downstream signaling molecules such as RAS play a role in cetuximab resistance of HNSCC. Since tumors harboring activating RAS mutations do not respond to EGFR-targeted therapy, it is important to screen RAS mutations before cetuximab treatment. Braig et al. investigated plasma ctDNA in liquid biopsy cohort of 20 HNSCC patients treated with cetuximab and found 6 of 20 patients (30%) acquired KRAS, NRAS or HRAS mutations [28]. Detection of RAS mutants may help to tailor anti-EGFR therapy as these mutants correlate significantly with treatment efficacy and disease progression. More importantly, mutational loads should be monitored during the therapy to reliably predict a loss of response to cetuximab. Thus, CTCs may be used for mutational monitoring to guide treatment decisions.

Detection of CTCs expressing programmed cell death ligand 1 (PD-L1) on their surface can be predictive of response for anti–PD-1 immunotherapy as PD-L1 is a key factor that suppresses T-cell function [29]. In breast cancer, Mazel et al. reported that PD-L1–expressing CTCs were detected in 11 of 16 (68.8%) patients, suggesting its usefulness in treatment planning [30]. PD-L1 expression was also reported in other tumor types such as lung [31], bladder [32], prostate and colorectal cancers [33], and was significantly associated with poor survival. In head and neck cancer, Strati et al. found PD-L1 expression in CD45-EpCAM+ (CD45-negative, EpCAM-positive) CTCs in 24 of 94 patients (25.5%) before treatment, 8 of 34 (23.5%) after chemotherapy, and 12 of 54 (22.2%) at the end of treatment [34]. Oliveira-Costa et al. investigated PD-L1 expression in OSCC-derived CD45+CK- CTCs and found

transcriptional and protein expression of PD-L1, suggesting its usefulness for monitoring patient's treatment response [35]. Moreover, Kulasinghe et al. isolated CD45-EpCAM+CK+ (CD45-negative, EpCAM-positive, Cytokeratin-positive) CTCs from a laryngeal cancer patient and detected high levels of PD-L1 by immunocytochemistry [36].

However, detecting CTCs is challenging due to their extremely low levels. It is estimated that only 1 to 2 CTCs are present per 7.5 mL of blood, making them difficult to study [21]. Currently, the only Food and Drug Administration (FDA)–approved platform for isolating CTC is CellSearch [37]. CellSearch is a standardized, semiautomated system that enables positive selection of CTCs based on the expression of the epithelial marker EpCAM. Testing therapeutic targets on a small population of CTCs in patients with HNSCC is currently under investigation, but its clinical utility has not yet been established.

2.3. Monitoring Treatment Response

ctDNA can be used in monitoring response to cancer treatment. Compared with imaging, ctDNA offers the diagnostic advantage of real-time monitoring of treatment response [38]. A recent study reported an early spike in plasma ctDNA levels (increase in BRAF mutated DNA) in melanoma patients with T-cell transfer immunotherapy, reflecting transient tumor cell death [39]. Another study found a reduction in ctDNA levels (EGFR mutations) in lung cancer patients after tyrosine kinase inhibitor (TKI) therapy, suggesting early indication of treatment response [40]. A gatekeeper mutation (EGFR T790M) associated with TKI resistance was also detected during ctDNA monitoring. Moreover, ctDNA is more sensitive than CTCs or cancer antigen 15-3 (CA15-3) as a circulating biomarker for predicting treatment response in breast cancer [41]. Thus, detecting the differential early dynamics of mutations may predict treatment response in the context of systemic therapy, enabling earlier therapeutic intervention.

A clearance study investigating the half-life of plasma EBV DNA in nasopharyngeal carcinoma patients demonstrated that the median half-life during chemotherapy was 3.99 d (range, 1.85–28.29 d) [42]. Another study reported that the half-life of plasma ctDNA (APC, KRAS, TP53, and PIK3CA) in colorectal cancer was 114 min after surgery, suggesting that ctDNA is an ideal biomarker to monitor rapid changes of tumor size because of its fast dynamics [12]. These findings suggest that viral DNA or ctDNA measurements could be used to reliably monitor tumor dynamics in cancer patients undergoing chemotherapy and/or surgery (Fig. 2A). Currently, a clinical trial to evaluate ctDNA as a biomarker for treatment response in head and neck squamous cell carcinoma is ongoing and the results are awaited (ClinicalTrials.gov Identifier: NCT03540563).

2.4. Monitoring Minimal Residual Disease

Recent studies have demonstrated that ctDNA levels can be exploited to monitor minimal residual disease (MRD) following surgery [43]. In principle, detection of ctDNA may be a more suitable approach than other circulating biomarkers for measuring MRD, as ddPCR has the highest sensitivity. Diehl et al. detected mutations as low as 0.01% in cell-free DNA in colorectal cancer patients, and those with MRD relapsed within 1 year after surgery [12]. A

prospective study of 230 patients with colorectal cancer demonstrated that relapse-free survival at 3 year after surgery was 90% for the ctDNA-negative group and 0% for the ctDNA-positive group [44]. In a separate study of 55 patients with breast cancer, postoperative ctDNA detection predicted poor relapse-free survival with a high level of accuracy [45]. Hamana et al. reported that ctDNAs were detected postoperatively by the use of microsatellite markers, predicting relapse in oral SCC [46]. Stratification of patients into high- or low-risk groups on the basis of ctDNA levels would enable earlier rescue treatment after surgery. Although there is evidence to indicate that ctDNA is a promising biomarker for monitoring MRD, whether identifying MRD-positive patients could improve patient outcomes through early therapeutic intervention remains to be elucidated in large clinical trials.

2.5. Predicting Metastasis

The current use of ctDNA is based on the evidence that it shares common mutational profiles with primary or secondary tumors. This implies that we can obtain a signature of metastasis without the need for an invasive tissue biopsy [11, 41]. In support of this, numerous studies have reported ctDNA to be a highly sensitive biomarker for metastasis, reflecting tumor burden and heterogeneity in various cancers, including head and neck cancer [11, 41, 47].

The prognostic value of CTC enumeration has also been demonstrated in various tumor types via large clinical trials [48-50]. There is growing evidence that detection of CTCs correlates with poor survival in head and neck cancer patients [23, 24, 51, 52]. In addition, CTC number was reported to be correlated with a higher incidence of regional metastasis in head and neck cancer [53]. However, these studies were unable to provide threshold CTC values correlating with poor prognosis as CTC numbers are highly variable among individuals. Although the clinical value of CTC analysis remains controversial, there is evidence indicating that CTC numbers after surgery or systemic therapy can be predictive of treatment outcomes and metastasis [54].

The low numbers of CTCs make their detection challenging. CellSearch method selects for tumor cells expressing EpCAM; therefore, downregulation of EpCAM during the epithelial-mesenchymal transition may make CTCs undetectable. Actually, only one-third of CTCs are found to be EpCAM positive in metastatic breast cancer patients [55]. This limitation might be overcome by combining different technologies and using additional markers. With this in mind, we propose that the combined use of ctDNA and CTCs may be an ideal strategy to assess the risk for metastasis in head and neck cancer.

3. Circulating Exosomal miRNAs

Exosomes are small, cell-secreted vesicles that carry diverse cellular constituents from their parental cells, including DNA, RNA, and protein. When secreted by a donor cell and then taken by an accepting cell, exosomes have important roles in exchanging molecular information between cells [56]. Given their content, exosomes could potentially be exploited as cancer biomarkers. Within the complex cargo of exosomes, miRNAs are the most relevant constituents for cancer diagnosis as they are regulatory molecules of both oncogenes and tumor suppressor

genes [57]. miRNA profiles in plasma exosomes have been reported to correlate with those in tumors from which they originate [58, 59]. These characteristics make exosomal miRNAs promising biomarkers for cancers [60-62]. The use of miRNA signature as a diagnostic tool in head and neck cancer has been explored previously. Summerer et al. reported that high expression of circulating miR-142, miR-186, miR-195, miR-374b, and miR-574 represent prognostic biomarkers for head and neck cancer [63]. Similarly, elevated levels of miR-21 and miR-24 were detected in plasma from head and neck cancer patients [64, 65]. Moreover, amplified miR-31 was detected in the plasma of head and neck cancer patients and was observed to have reduced after tumor resection, suggesting its tumor origin [65]. Down-regulation of tumor-suppressive miR-486 was highly associated with OSCC recurrence, suggesting miR-486 could act as a biomarker to monitor OSCC recurrence after surgery [66]. These reports provide considerable evidences that exosomal miRNAs can be exploited as a valuable tool in cancer diagnosis.

The miRNA database, miRandola, provides a comprehensive catalog of extracellular noncoding RNAs identified in various diseases, including cancer, and currently contains 3,283 entries with 1,002 miRNAs (http://mirandola.iit.cnr.it/) [67]. Although a number of exosomal miRNAs have been proposed as diagnostic and prognostic markers, most have been investigated by inconsistent methods, and the heterogeneous results hamper the reliability of miRNAs in clinical diagnosis [68]. Moreover, it is unclear whether immune cells make a strong contribution to circulating miRNA levels. Systemic or local inflammation may perturb miRNA expression and its reproducibility even within the same individual [69]. The diagnostic performance of exosomal miRNAs in head and neck cancer remains inconsistent among studies; thus, more studies are needed to further characterize exosomal miRNAs. Validation in large clinical trials with standardized protocols is required to substantiate the value of exosomal miRNAs in a clinical setting.

4. Salivary diagnostics

In the past decade, saliva researchers have developed salivary diagnostics to detect oral and systemic diseases [70]. This simple and inexpensive method, allows the recollection of biological samples, revealing specific biomarkers associated with health or disease. Proteomic studies of saliva revealed that 20–30% of the salivary proteome mirrors the plasma proteome, indicating that a substantial portion of salivary constituents are derived from the blood [71, 72]. The significant overlap between saliva and blood due to their physiological interactions indicates a potential alternative approach to diagnosing systemic diseases. Saliva possesses several advantages over blood as a body fluid for clinical diagnosis. Saliva collection is performed easily and noninvasively, thereby reducing patient discomfort. Unlike blood, saliva does not coagulate, making it easier for handling and processing. Saliva is a readily available biofluid regarded as a mirror of oral and systemic health, containing a wide variety of biomarkers, rendering it an attractive biofluid for early disease detection.

4.1. Salivary ctDNA

Saliva contains cell-free DNA, and genomic analysis revealed that 70% of salivary DNA originates from the host and 30% from the oral microbiota [73]. Salivary ctDNA has been demonstrated to be a more sensitive biomarker than plasma ctDNA for early stage oral cancer (Table 1). A proof-of-principle study demonstrated that ctDNA can be detected in saliva in early stage oral cancer with 100% sensitivity [14]. Even in patients with cancers at other sites (oropharynx, hypopharynx, and larynx), ctDNA was found in the saliva of 47% to 70% of these patient groups, making it a valuable biomarker for detecting head and neck cancer.

Qureishi et al. examined the accuracy of PCR-based HPV DNA detection in saliva from OPSCC patients and found acceptable diagnostic accuracy with a positive predictive value of 96% [74]. The sensitivity and specificity of saliva testing when compared to the reference p16 immunohistochemistry (IHC) on surgical biopsies were 72.2% and 90%, respectively. Martin-Gomez et al reported a prospective study that confirmed oral gargle samples as reliable and noninvasive sources of HPV DNA in OPSCC patients [75]. Among 171 cases that had paired oral gargle sample and tumor specimens, concordance rate for HPV 16 was 74% and 94%, respectively. Regarding other HPV types (e.g. HPV 18, 31, 33, 35), concordance rates were even higher (> 94%). This demonstrates the feasibility of detection of HPV DNA in the saliva of head and neck cancer patients.

Another possible implication of oral HPV detection is monitoring disease after treatment for HPV-positive OPSCC. Ahn et al. reported a retrospective study that demonstrated a positive posttreatment saliva HPV status was associated with higher risk of recurrence (hazard ratio, 10.7; 95% CI, 2.36-48.50) (P = .002) [76]. Rettig et al. showed a similar result that evaluated the predictive value of HPV DNA in saliva [77]. In this prospective study, oral rinse samples from 124 patients with HPV-positive OPSCC before and after treatment (surgery or chemoradiotherapy) were collected. HPV DNA was present in 54% of oral rinse samples from patients prior to treatment but was seen in only 5% following treatment. Importantly, 5 of 6 patients (83%) with persistent HPV DNA in oral rinse samples developed recurrent disease, implicating saliva as a possible surveillance tool. Thus, saliva is enriched with tumor or viral-specific DNA originating from tumor cells in the oropharyngeal cavity. Analyzing both saliva and plasma may be optimal for effective screening of head and neck cancer (Fig. 2B).

4.2. Salivary miRNA

Circulating cell-free salivary miRNAs were first discovered and characterized in our laboratory [78]. Salivary miRNAs were found to be remarkably stable, and endogenous salivary miRNAs degrade at a much slower rate than exogenous miRNAs. miRNA profiling demonstrated that salivary miRNAs are packaged into exosomes, rendering them resistant to degradation by RNases [79, 80]. Since then, salivary miRNAs have been studied as potential biomarkers for head and neck cancer on the basis of their relative ease of collection and detection.

We reported that miR-125a and miR-200a were significantly less in saliva collected from oral cancer patients than they were in controls [78]. Similarly, the expression of miR-139 and

miR-375 was also found to be decreased in saliva collected from oral cancer patients compared with that obtained from normal controls [81, 82]. In addition, increased expression of miR-27b and miR-31 was observed in saliva obtained from oral cancer patients compared with that obtained from controls [83, 84]. Importantly, expression levels of miR-139 and miR-31 reverted to baseline after excision of the lesions, suggesting their potential use as prognostic biomarkers [81, 83]. Although certain sets of salivary miRNAs may serve as putative biomarkers for head and neck cancer, further research is required to validate these findings and elucidate the molecular mechanisms involved.

4.3. Salivary Exosomes

Salivary exosomes are naturally-occurring nanovesicles that are secreted from salivary gland and oral epithelial cells into saliva. Salivary exosomes are surrounded by a phospholipid bilayer and carry a unique cargo of proteins and nucleic acids that can reflect those of the cell of origin (Fig. 3). The most commonly detected proteins are tetraspanins (e.g., CD63, CD9, and CD81), heat shock proteins (e.g., Hsp70 and Hsp90), water channel, major histocompatibility complexes (MHC class I), membrane transporters and fusion proteins (e.g., Rab GTPases and annexins), and ESCRT (endosomal sorting complex required for transport)-associated proteins (e.g., Alix and Tsg101) that are involved in exosome biogenesis from MVBs [85, 86]. Other proteins found on exosomes include signaling, cytoskeletal, metabolic, and carrier proteins. Numerous proteomic studies on mammalian EVs have yielded extensive catalogues of proteins found in various types of EVs isolated from cells, tissue, or body fluids. The databases are publicly available at Vesiclepedia (http://www.microvesicles.org) [87] and ExoCarta (http://www.exocarta.org) [88]. Both databases include data not only on proteins but also on nucleic acids and lipids, as well as on the constituents isolated from salivary exosomes.

Elucidating the nanostructural differences between the salivary exosomes originating from healthy subjects compared to patients with disease is particularly important as disease-specific exosomes may differ in functional properties [89]. In a nanostructural characterization study, the salivary exosomes from healthy donors using atomic force microscopy (AFM) and field emission scanning electron microscopy (FESEM) identified 70–100-nm exosomes with trilobed structures, demonstrating their reversible and elastic mechanical properties [90, 91]. Low-force imaging revealed round-shaped exosomes, suggesting exosomes have an inherent spherical morphology when stresses are not applied. Additionally, AFM phase contrast images portrayed exosomes with a heterogeneous surface, likely attributed to the embedded proteins in a dense lipid membrane.

Morphological characterization of salivary exosomes at the single-vesicle level using high-resolution AFM displayed irregular morphologies and higher intervesicular aggregation in an oral cancer patient compared to a healthy control [92]. Quantitative analysis also revealed that size and CD63 surface density were significantly increased in cancer exosomes (98.3 ± 4.6 nm) compared to normal exosomes (67.4 ± 2.9 nm) ($p < 0.05$). Structural and morphological aberrations in the exosomes are suggestive that these exosomes are at least in part cancer-derived products that were shed directly into saliva. Multivesicular bodies (MVBs) were

identified in oral cancer salivary exosome fractions [92]. These multivesicular structures showcased ruptures and elongated nanofilaments around the lumen of these MVBs, suggesting that these are the sites for exosome release, as well as filamentous extension of nucleic acids (Fig. 4). These images suggest that oral cancer-derived exosomes in saliva have distinct properties that make them potential biomarkers for cancer diagnosis.

5. Conclusions and Future Perspectives

A growing body of evidence implicates the clinical utility of circulating biomarkers extracted from multiple body fluids for cancer patients, focusing on patient stratification and monitoring disease status. Actively released ctDNA in plasma and saliva may be preferred for the early detection of head and neck cancer, whereas CTCs released from metastatic lesions may predict poor prognosis. Analyzing CTCs for surface expression of drug targets such as EGFR and PD-L1 can provide critical information for planning immunotherapy. Other circulating biomarkers such as exosomal miRNAs can provide additional layers of information; thus, targeting multiple types of biomarkers that have independent mechanisms of release may increase the specificity and sensitivity of cancer diagnosis.

The key question concerning all types of circulating biomarkers (ctDNA, CTCs, and exosomal miRNAs) is how representative they are of the whole tumor. In this regard, assessment of biomarkers should be considered in the context of integrity and inclusivity of all tumor features. For example, mutated DNA fragments such as TP53 and PIK3CA can only provide information about limited regulatory pathways. CTCs are considered to contain the complete cellular information; however, if a tumor is heterogeneous, they might represent just a small proportion of the tumor, thereby compromising their clinical relevance. In contrast, exosomes are expected to represent more of the tumor because they are thought to be derived from the whole tumor, reflecting its heterogeneous characteristic. However, the cargo of exosomes has been demonstrated to be selectively assembled through the trans-Golgi network and endosomal system; therefore, exosomal miRNAs can be only partially representative of whole cellular miRNAs. Further studies with large patient cohorts using standardized protocols are required to determine whether each approach, individually as well as combined, can improve overall survival.

Analysis of circulating biomarkers in multiple body fluids alongside plasma can provide complementary information and represent key milestones toward the implementation of liquid biopsies in personalized medicine. The use of saliva is easy, noninvasive, and informative; thus, salivary diagnostics may fulfill the ambitions of precision medicine initiatives.

We first coined the term saliva-exosomics to describe next-generation salivaomics that studies salivary exosomes through the advanced “omics” technologies to better delineate their specific functions and biomarkers for cancer [70]. A unique subset of exosomes originating from tumors appears to be present in saliva, which might be due to processing and selection of tumor-derived exosomes in the salivary gland (Fig. 5). Nevertheless, the amount and content of salivary exosomes are highly variable even in patients with the same tumor types and stages. A

more in-depth understanding of the biology of salivary exosomes will provide the basis for biomarker development and new therapeutic avenues.

Establishing simple, rapid, and affordable technologies to analyze circulating biomarkers represents an important future challenge. Currently, ddPCR and next-generation sequencing play a central role in ctDNA and miRNA analysis. However, high cost and complicated data manipulation impede their applications in routine clinical care. We recently developed a novel diagnostic platform, EFIRM (electric field-induced release and measurement), that can detect ctDNA and exosomal RNA directly, only requiring 40 μL of body fluid [93-95]. Further development and application of saliva-based point-of-care technologies will allow for better immediate clinical management, leading to earlier intervention and reduced morbidity and mortality.

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