Liquid Biopsy and Oral Cancer Management

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Mouth cancer often results in poor outcomes and requires the use of state-of-the-art medical approaches to make its detection easy, individualized, and early. Liquid biopsy is a new and important medical approach to disease detection. This approach has been successfully used for mouth cancer detection and monitoring of treatment progress in many countries. Liquid biopsy is an attractive option for mouth cancer detection because it does not involve any invasive procedure and can be used on easily accessible body fluids, such as saliva and blood.

Keywords: Africa ; cfDNA ; circulating tumour cells ; exosomes ; liquid biopsy

1. Introduction

Reaching the goal of early and accurate diagnosis, as well as providing the best evidence-based treatment option for disease, is a key driving force for precision and individualized medicine [1]. A plethora of diagnostic methods have already been developed and, recently, liquid biopsy (LB) has shown increased global interest as a precision diagnostic tool in the field of cancer research. Cancer as a major global public health problem is emerging as a critical concern in Africa, where many cancer cases are diagnosed at late stages of the disease. This is due to factors such as limited knowledge and expertise of disease screening, limited diagnostic infrastructure, and for most patients, fear of surgery, poverty, lack of access to specialist care, and low educational level, among other factors—these are some of the key barriers to early presentation and cancer diagnosis among African populations [2].

Novel diagnostic tools such as LB could help in addressing these challenges due to its non-invasive nature, accuracy, and the fact that it does not require surgical facilities. LB has emerged as a rapid, reliable, and minimally invasive cancer screening solution, with high specificity and sensitivity for cancer diagnosis and monitoring. As demonstrated in developed countries, the high specificity and sensitivity of LB offers a promising diagnostic tool that would enhance screening capability and the potential for the early diagnosis of cancer cases in Africa; as well as probably reduce the incidence of morbidities and mortalities from cancer on the continent. In addition, the implementation of policies that allow easy access to valuable cancer diagnostic procedures, such as LB, can further help reduce the financial burden of late cancer management in many low-income countries in Africa.

Oral squamous cell carcinoma (OSCC) ranks amongst the ten most prevalent cancers in the world with high morbidity and mortality rates [3]. This emphasizes the need for, and the importance of, screening programs and techniques for the early detection of malignancy. A lack of access to oral health care, which can lead to a delay in diagnosis has been reported to decrease the survival rates of OSCC in several low and middle-income countries including those in Africa [4]. The timely detection and diagnosis of OSCC may save lives by improving the survival rate, reducing treatment-related morbidities and improving the surveillance of recurrent cancer cases in these countries [5].

2. Liquid Biopsy and Cancer Management

For centuries, the use of tissue biopsies in cancer has enabled the histological characterization of the disease. Its application has provided insights into the genetic profile of tumors, allowing for good cancer management [6]. Notably, tissue biopsy remains the gold standard for diagnostic analyses in clinical settings. However, tissue biopsies involve invasive surgical techniques, cost, and tissue sample preparation. More importantly, tissue biopsies may not capture genetic heterogeneity within a tumor, and in intermetastatic tumor samples, thus affecting the accuracy of the test [7]. These challenges described make LB an appealing alternative, especially as a useful instrument in long-term management and prognostication.

LBs can be used to investigate biological components in liquid forms in cancer patients for diagnosis, screening, and prognosis. LB may involve the analysis of released circulating tumor cells (CTCs) and circulating tumour DNA (ctDNA) in the blood or body fluid of a cancer patient [8]. These analytes are complementary biomarkers that present great potential

for various cancer drug discovery platforms. Other analytes that can also be identified by using LB include circulating cell-free RNA (cfRNA), exosomes and platelets [9]. Liquid biopsy analytes can improve their understanding of tumor heterogeneity, and provide potentially better cancer diagnosis, treatment, and surveillance, as well as detecting drug resistance. Importantly, saliva, urine, pleural effusions, seminal plasma, sputum, cerebrospinal fluid, and stool samples are other physiological fluids that can be utilized for a LB in addition to blood [10][11].

2.1. Circulating Cell-Free DNA (cfDNA) and Circulating Tumor DNA (ctDNA) in OSCC

Fragmented, tumor-derived DNA that are not associated with cells (i.e., cell-free) and which are found within the circulatory system are known as ctDNA. They are the tumor derived part of circulating cell-free DNA (cfDNA), which refers to the total DNA shed into the blood and biological fluids during apoptosis and necrosis under physiologic and pathologic conditions [12]. Currently, ctDNA has been used in monitoring the therapeutic response and the detection of cancer relapse at early stages. Regarding treatment response and relapse detection, the identification of tumor-specific point mutations, promoter hypermethylations, and the identification of allelic imbalance using microsatellite markers analysis in ctDNA are helpful tools of assessment in patient management [13]. The ctDNA possesses short nucleic acid fragments of around 166 bp located in the plasma [14]. Healthy individuals have lower levels of cfDNA when compared with OSCC patients, which further increase as the cancer metastasizes, indicating its simultaneous use as a promising diagnostic and prognostic biomarker [15]. However, increased cfDNA is not specific for cancer, and in individual patients, there is no specific cut-off value which can be attributed to the tumor in quantification as ctDNA. This limitation can be overcome by evaluating tumor specific alterations such as methylations and mutations [16]. The potential for using ctDNA in all stages of head and neck squamous cell carcinoma (HNSCC) diagnosis and management was highlighted in a recent review—this included screening and early detection; prognosis and the detection of minimal residual disease; the characterization of mutational landscape; precision medicine and treatment selection; and treatment monitoring and identification of resistant clones, based on experience with HNSCC of other sites using ctDNA analysis [17]. Several studies have been developing techniques to detect cfDNA (or ctDNA), but there is still a lack of a standardized method, which is essential for its clinical application together with the need to reduce the cost of analysis. Studies have identified ctDNA as a diagnostic biomarker and this has been used in many diseases [18]. Although these studies demonstrated that circulating cfDNA can screen for cancer; when testing for ctDNA, the procedure requires additional investigation and an acceptable cost before these can be applicable for use as a diagnostic or therapeutic tool, especially in African and other resource-limited settings.

2.2. Circulating Tumor Cells (CTCs) in OSCC

CTCs are released into circulation by the primary tumor (i.e., they are of tumor origin) as a result of spontaneous or iatrogenic factors. They are believed to share a similar profile with the somatic mutations and genomic rearrangements present in the primary tumor [19]. They are able to reflect the tumor heterogeneity, which may be missed in surgical biopsies. This makes them good candidates for understanding tumor mutational profiles without allowing patients to go through invasive tissue biopsy. A major problem with CTC's use in patient management is their extremely low number in peripheral circulation (1–100 CTCs per 1 billion peripheral blood cells). They need to be enriched and separated for detection and analysis by often tedious and expensive methods [20][21]. Increasing levels of CTCs have been reported to be associated with poor prognosis and distant metastasis in several forms of cancer. Survival rate in OSCC, and other cancers such as breast, lung, prostate and ovarian cancer, has been linked with levels of CTCs, indicating their benefit in the early screening and treatment monitoring of cancer [22]. Partridge et al. [23] evaluated the levels of disseminated tumor cells preoperatively and intraoperatively in both blood and bone marrow from 40 patients with OSCC. They found a high risk of loco-regional recurrence and distant metastasis associated with the presence of CTCs. In 2015, Oliveira-Costa et al., provided more knowledge regarding CTC biology for OSCC by analyzing the gene expression profile of OSCC tumors to identify biomarkers that decreased or increased during tumor progression [24]. Their results showed that programmed death-ligand 1 (PD-L1), HOXB9 and ZNF813 expression in OSCC-derived CTCs was increased, while B Cell Linker (BLNK) expression decreased. In summary, the investigators reported that PD-L1 is a prognostic factor in OSCC as expressed in patients CTCs and provides insights for the development of an anti-PD-L1 therapy for OSCC patients. Due to PD-L1 inducing an exhaustion state in T-cells and reducing the capability of a T-cell-mediated response, they hypothesized that OSCC patients could benefit from anti-PD-L1 therapy. Therefore, the results reported in this entry emphasized the role of CTCs as an independent prognostic marker in OSCC. Recurrent assessments of CTC levels have also been used in studies to demonstrate the potential utility of CTCs in disease monitoring before, during, and after therapy, Inhestern et al. [25] examined and evaluated CTC counts in blood samples from 40 patients with OSCC. Apart from its potential as a prognostic biomarker, there is interest in investigating the role of CTCs in regulating disease behavior. The checkpoint inhibitors that block the PD-1/PD-L1 immune checkpoint pathway on CTCs and stimulate the immune system to remove CTCs in circulation may reduce the risk of metastasis and disease recurrence. In patients with OSCC, PD-L1 overexpression in CTCs was identified and utilized to monitor the treatment response [24]. Numerous

studies [26][27][28][29][30] showed that LB procedures, such as CTCs, can be utilized as a cutting-edge technology that may improve the detection and monitoring of cancer using a small amount of blood samples. CTCs assessment using LB as a cancer biomarker promises to be low cost for the management of patients with OSCC.

2.3. Exosomes in OSCC

Exosomes are bioactive vesicles with diameters ranging between 40–150 nm that are used for analyzing LBs. A miRNA expression profile has shown that circulating exosomal miR-21 was associated with hypoxic tumor and metastasis in the lymph node in people with OSCC [31]. The detection of miRNA biomarkers in both the plasma and tumors of patients with squamous cell carcinoma of the tongue highlights the significance of free and exosomal miRNAs as potential diagnostic biomarkers for tongue cancer. In addition, packaged circulating miRNAs in protein complexes or encapsulated within microvesicles are protected against the activity of blood RNAses, and represent a more dependable approach for the assessment of circulating tumor-miRNA signatures [10]. More so, exosomes in the tumor microenvironment have been implicated in increasing levels of the transforming growth factor-B (TGF-β) pathway; thus, increasing drug resistance and tumor growth in OSCC patients. Exosomal chemokine-like factor (CKLF)-like MARVEL transmembrane domain-containing 6 (CMTM6) of OSCC cells aid the polarization of alternatively activated macrophages (M2) via activation of the signaling of ERK1/2 in macrophages [32]. Indeed, the classically activated macrophages possess anti-tumor properties; conversely, the M2 functions as a pro-tumor, aiding in the development and progression of the tumor [33]. Exosomes are released by different types of cells into numerous biological fluids such as amniotic fluid, cerebrospinal fluid, lymph, bile, ascites, tears, breast milk, urine, semen, blood, and saliva, both in healthy and diseased conditions [34][35][36][37]. Two research groups have demonstrated that exosomes are present in the tumor microenvironment, demonstrating its importance in tumorigenesis, tumor invasion, and metastasis, since they can possess an anti-tumor function or promote tumor progression [38][39]. In OSCC, exosomes have been shown to be key components in the tumour microenvironment, increasing the TGF signaling pathway, which contributes to the progression and drug resistance of OSCC [40]. Limited available evidence may suggest a potential discriminatory biomarker role of exosomes, between active OSCC disease patients and cured OSCC patients. In addition, a study by Zlotogorski-Hurvitz et al. [41] morphologically characterized oral fluid-derived exosomes in OSCC. The potential scope of the diagnostic and prognostic application of exosomes in oral cancer has been described elsewhere [32]. Additionally, the role of some exosomal miRNA (e.g., miR-223, miR-101-3p, miR-338 and miR-34a-5p) as tumor suppressors and the robust potential of exosomes for therapeutic drug delivery to the tumor for effective treatment or to improve prognosis has been highlighted in another recent review [34].

2.4. Messenger Ribonucleic Acid in OSCC

LBs find and evaluate several biomarkers, such as mRNA biomarkers, pro-inflammatory cytokines, and metabolites in the saliva, urine, and plasma of OSCC patients. mRNA biomarkers from saliva for use in the early diagnosis of oral cancers were recently described by Oh and colleagues [35]. Thirty candidate genes related to cancer previously reported in the literature were selected. Thirty-three OSCC patients and 34 non-tumor controls had their saliva samples taken and the mRNA levels of six genes CYP27A1, NAB2, collagen type III alpha 1 (COL3A1), monoamine oxidase B (MAOB), nuclear pore complex interacting protein B4 (NPIPB4), and sialic acid acetyltransferase (SIAE) were considerably lower in the saliva of OSCC patients. The combination of SIAE and CYP27A1 had an AUC of 0.84, which was considered good. In the under 60 group, the AUC of MAOB–NAB2 was more prognostic of OSCC (AUC, 0.91; specificity, 0.86; and sensitivity, 0.92) than any other transcript combination. The results from this entry suggested salivary mRNAs were useful biomarkers for early OSCC diagnosis, especially in individuals under 60 years old. Lu et al. [36] found substantially increased expression of plasma miR-196a and miR-196b in patients with cancerous and precancerous oral cavity lesions, with excellent sensitivity and specificity compared to the normal controls.

2.5. Saliva as a Liquid Biopsy Substrate

Saliva allows for a non-invasive LB; it is easily accessible and has a large number of biomarkers for illnesses, as well as pre-symptomatic and health status indicators. The saliva biofluid contains RNA and DNA molecules, cytokines, extracellular vesicles (EVs), and circulating and tissue-derived cells, which are novel biomarkers or indicators [38][39][40]. Salivary biomarkers may be effective for the early detection of OSCC since they are physically accessible to the mouth cavity [38]. In saliva and tissue samples from individuals with OSCC and oral potentially malignant diseases, NF-κB-dependent cytokines, and pro-inflammatory cytokines (IL-6, IL-1α, IL-8, and TNF-α) were assessed [41]. NF-κB-dependent cytokines, matrix metalloproteinases (MMPs) and pro-inflammatory cytokines in saliva may be involved in the relationship between oral malignancies and aging, involving the senescence-associated secretory phenotype (SASP), and inflammatory diseases, such as oral mucosal ulcers and periodontitis [42].

2.6. Novel Molecular Techniques for Application of Liquid Biopsy

More sensitive emerging technologies are now employed for LB analysis. These technologies include beads, emulsion, amplification, and magnetics (BEAMing), digital droplet PCR (ddPCR) and next generation sequencing (NGS) [36]. In respect of experimental and clinical applications, BEAMing, ddPCR and NGS have separate applications, and they sometimes complement each other for the examination of a tumor at molecular level [36]. ddPCR is very reliable and accurate for the investigation of genetic alteration in various cancers due to its very sensitive nature [37]. However, this method is challenged by a low multiplexing capacity, but efforts are in the pipeline towards designing multiplexed strategies to reduce experimental artefacts [37]. LBs can be used to monitor OSCC treatment responses and tumor evolution in real-time and improve the prognostic and diagnostic potential for OSCC [10][38]. One of the essential benefits of analyzing LBs in OSCC is that they give a tailored snapshot of primary and metastatic tumors at different periods, allowing clinicians to detect early signs of disease recurrence or resistance to therapy and assisting them in their therapeutic decisions [10]. As a result, by employing LBs, a molecular profile for each patient can be acquired. Different tumor subtypes may be useful in supplementing the tumor, node, and metastasis (TNM) staging approach [10]. A summary of the applications of LB in OSCC can be found in **Table 1**.

Table 1. Some evidence of the applications of liquid biopsy in oral cancer.

Study	Country	Type of Cancer	Liquid Biopsy Technology	Publication Type	Reference
Lousada-Fernandez et al., 2018	Spain	Oral cancer	Liquid Biopsy	Review	[10]
Lin et al., 2018	Taiwan	Oral Cancer	Cell-Free DNA Biomarker	Original Research	[15]
Patel et al., 2016	India	Oral Squamous Cell Carcinoma	Circulating Tumor cells	Original Research	[19]
Economopoulou et al., 2017	Greece	Head and Neck Squamous Cell Carcinoma	Circulating Tumor Cells	Review	[20]
Oliveira-Costa et al., 2015	Brazil	Oral Squamous Cell Carcinoma	Circulating Tumor Cells	Original Research	[24]
Inhestern et al., 2015	Germany	Oral and Oropharyngeal Squamous Cell Cancer	Circulating Tumor Cells	Original Research	[25]
Li et al., 2016	China	Oral Squamous Cell Carcinoma	Exosomes	Original Research	[31]
Pang et al., 2021	China	Oral Squamous Cell Carcinoma	Exosomes	Original Research	[32]
Lu et al., 2021	China	Oral Squamous Cell Carcinoma	Exosomes	Review	[34]
Oh et al., 2020	South Korea	Oral Cancer	Salivary mRNA Biomarker	Original Research	[35]
Lu et al., 2015	Taiwan	Oral Cancer	Circulating miRNA Biomarker	Original Research	[36]
Liu et al., 2010	Taiwan	Oral Cancer	Circulating miRNA Biomarker	Original Research	[37]
Cristaldi et al., 2019	Italy	Oral Squamous Cell Carcinoma	Salivary Biomarker (ctDNA, EVs and miRNAs)	Review	[38]
Adeola et al., 2020	South Africa	Oral Cancer	Salivary Exosomes Biomarker	Review	[40]
Tsai et al., 2020	Taiwan	Oral Squamous Cell Carcinoma	Nuclear Magnetic Resonance Metabolomics Biomarker	Original Research	[43]
Ono et al., 2018	Japan	Oral Cancer	HSP-Enriched Properties of Extracellular Vesicles	Original Research	[44]
Fujiwara et al., 2018	Japan	Oral Cancer	Exosomes	Original Research	[45]

Study	Country	Type of Cancer	Liquid Biopsy Technology	Publication Type	Reference
Spafford, et al., 2001	USA	Head and Neck Squamous Cell Carcinoma	Pretreatment Oral Rinse Microsatellite Analysis.	Original Research	[46]

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