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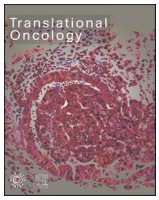

Saliva as a potential non-invasive liquid biopsy for early and easy diagnosis/prognosis of head and neck cancer

Prabhat Kumar [1], Shilpi Gupta [1], Bhudev C. Das [*]

Stem Cell and Cancer Research Lab, Amity Institute of Molecular Medicine & Stem Cell Research (AIMMSCR), Amity University Uttar Pradesh, Sector-125, Noida 201313, India

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A B S T R A C T

Head and neck squamous cell carcinomas (HNSCCs) are the most devastating diseases in India and southeast Asia. It is a preventable and curable disease if detected early. Tobacco and alcohol consumption are the two major risk-factors but infection of high-risk HPVs are also associated with development of predominantly oral and oropharyngeal carcinomas. Interestingly, unlike cervical cancer, HPV-induced HNSCCs show good prognosis and better survival in contrast, majority of tobacco-associated HPV^{-ve} HNSCCs are highly aggressive with poor clinical outcome. Biomarker analysis in circulatory body-fluids for early cancer diagnosis, prognosis and treatment monitoring are becoming important in clinical practice. Early diagnosis using non-invasive saliva for oral or other diseases plays an important role in successful treatment and better prognosis. Saliva mirrors the body's state of health as it comes into direct contact with oral lesions and needs no trained manpower to collect, making it a suitable bio-fluid of choice for screening. Saliva can be used to detect not only virus, bacteria and other biomarkers but variety of molecular and genetic markers for an early detection, treatment and monitoring cancer and other diseases. The performance of saliva-based diagnostics are reported to be highly (≥95 %) sensitive and specific indicating the test's ability to correctly identify true positive or negative cases. This review focuses on the potentials of saliva in the early detection of not only HPV or other pathogens but also identification of highly reliable gene mutations, oral-microbiomes, metabolites, salivary cytokines, non-coding RNAs and exosomal miRNAs. It also discusses the importance of saliva as a reliable, cost-effective and an easy alternative to invasive procedures.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the major global health problem and 6th most common cancer worldwide, and the highest incidence and mortalities are reported in Southeast Asian countries [1]. Globally, HNSCCs account for 1536,031 new cases and ~1011,202 deaths annually [2]. As per GLOBOCAN 2020 report, Indian HNSCC statistics indicate that there are 296,449 new cases per year and approximately 188,713 HNSCC related annual mortalities, making it a second most common cancer after breast cancer in India [2] (Fig. 1). HNSCCs are highly heterogenous group of diseases which develop through multistep process from normal mucosa to precancerous lesions including leukoplakia, erthroplakia and dysplasia to invasive cancer [3]. The major symptoms of the disease are chronic pain, altered voice and facial look, speech difficulty, dysphagia and social isolation. The key risk-factors for HNSCC development are tobacco chewing, smoking, alcohol intake, genetic alterations, poor oral hygiene, sexual behavior, poor immunity and infectious agents such as Human papillomavirus (HPV), Epstein-barr virus (EBV) and herpes simplex virus type 1 (HSV1) [4,5].

Despite the improvement in surgical and therapeutic approaches and ease of access to clinical examination and apparent early warning signs and symptoms, patients lose the opportunity for early diagnosis and therapy and showed worst survival rate. The overall five-year survival rate (OSR) is still ~50 % [6]. Furthermore, the poor prognosis rate of HNSCCs is also associated with tumor invasion, recurrence, aggressive metastasis and lack of specific molecular biomarker(s) for early detection [7]. Therefore, there is an urgent need to identify novel molecular

* Corresponding author at: Stem Cell and Cancer Research Lab, Amity Institute of Molecular Medicine & Stem Cell Research (AIMMSCR), Amity University Uttar Pradesh, Sector-125, Noida 201313, India.
E-mail address: bcdas@amity.edu (B.C. Das).
[1] Authors contributed equally.

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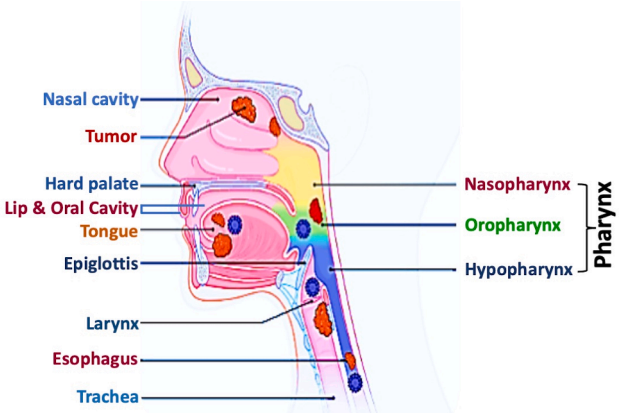

Fig. 1. Location of different anatomical sites of head and neck region that are prone to develop cancer.

biomarker(s) associated with the development of HNSCC, patient's diagnosis and prognosis. Currently, tissue biopsy and histopathological-based diagnosis are the gold-standard methods for cancer screening and diagnosis. However, these methods have various limitations such as patient's physical and functional distress due to invasiveness, time-taking, expressive and need a trained professionals. Liquid biopsy-based molecular biomarker detection offering a safe, non-invasive and reliable method than standard invasive methods for early disease diagnosis, prognosis and treatment monitoring. Liquid biopsies such as blood, serum, urine and saliva, are highly attracting procedures for cancer management and to track potential cancer-related heterogeneity and treatment efficacy [8–11]. Numerous potential biomarkers are being investigated to predict clinical outcome and provide individualised treatment of patients with HNSCC. Although several biomarkers have been discover over the years using human blood, biopsy and non-invasive saliva but implementation of many these biomarkers in routine clinical practice is still awaited [12] to improve contextualize the issues in diagnosis and treatment of head and neck cancer patients.

Saliva has a lot of potential as a most convenient non-invasive diagnostic fluid, and has huge advantages over other biological samples such as blood, smears and tissue biopsies in terms of cost, ease of collection, multiple sampling possibilities and reducing risk of infectious agents being transmitted to healthcare workers, for monitoring systemic health, disease progression and prognosis. Salivary biomolecules can be used as reliable diagnostic tools to detect a number of malignancies, genetic diseases, and hormonal abnormalities. Salivary diagnostics can also help to detect oral infections, oral microbiome, metabolites, gene mutations, epigenetic alterations, cytokines, immunoglobulins, hormones, mucins, growth factors, inhibitors, enzymes, non-coding RNAs and salivary exosomes and exosomal miRNAs [13–17]. Thus, saliva is considered as a therapeutically invaluable bio-fluid as it contains variety of important biological molecules and macromolecules which can easily serve as biomarkers for disease diagnosis, prognosis and therapeutic targets for efficient treatment and monitoring of patients for both oral and other systemic diseases. Salivary testing is the easiest of all the currently testing methods since it does not cause any discomfort, pain, toxicity, inconvenience, and cost-effective and widely used as most common screening procedure.

In this article, the authors focus on the emerging potential of saliva as a micro-fluidic diagnostic tool for an easy detection and clinical surveillance of oral diseases including oral and other head neck cancers at an early stage. The article also summarizes diagnostic potential of saliva for detection of other infectious agents without direct contact with the patient and monitoring disease progression, prognosis including monitoring of vaccines efficacy.

HNSCC subsites specific incidence and mortalities

HNSCCs encompass a wide spectrum of heterogeneous tumors originating from the mucosa of the lip, oral cavity, nasal cavity, oropharynx, nasopharynx, larynx, hypopharynx and salivary glands [18]. The major histological type of HNSCC is squamous cell carcinoma (SCC), constituting about 90–95 % of head and neck malignancies and 5–10 % are adenocarcinomas [19]. Each HNSCC subsite exhibits distinct etiology, epidemiological factors, genetical, molecular and clinical characteristics including treatment outcome [20]. In India, the distribution and/or incidence of HNSCC at various subsites are distinct, 36 % for lip & oral cavity, 21 % oropharynx, 4.2 % for nasopharynx, 18.8 % for larynx, 33.8 % for hypopharynx and 14.7 % for salivary glands (see Fig. 1 and Table 1) [2]. Worldwide, cancers of oral cavity, larynx and oropharynx are the most prevalent head and neck cancers. Surprisingly, more than 40 % of HNSCC associated mortalities occur due to lip and oral cavity cancer which is the number one cancer in Indian males (Fig. 1) [21,22]. Cancers in these subsites differ with respect to specific risk-factors, symptoms, stage, tendency to local and distant metastasis, sensitivity to chemo-radiotherapy and the disease prognosis [23,24], suggesting that subsite specific analysis could be an independent prognostic factor for HNSCC.

Components of saliva and their physiological functions

Saliva is a hypotonic bio-fluid that could be a good source of biochemical markers in identifying specific diseases, making it valuable for novel approaches to understand prognosis, laboratory or clinical diagnosis, as well as monitoring and care of patients with oral or other systemic disorders. Saliva has several advantages as a diagnostic bio-fluid since it is easy to collect, non-invasive, safe, easy to preserve and contains adequate quantity of genetic materials [25]. Saliva is primarily secreted from three glands: the parotid, submandibular, and sublingual and other minor salivary glands and a mixture of crevicular fluid, bronchial and nasal secretions. It may also contain blood derivatives from oral wounds, microorganisms, exfoliated epithelial cells, and food debris. Saliva is slightly acidic (pH=6–7) and every day, a healthy person produces ~1–1.5 liter of saliva, at a rate of about 0.5 mL per minute [26].

Saliva with versatile physical properties contains ~98 % water and 2 % organic and inorganic substances. It is a complex biological fluid that contains variety of DNA, RNA, miRNAs, proteins, hormones, growth factors, inhibitors, enzymes, minerals, electrolytes, buffers, immunoglobulins (IgA), antimicrobial components, cytokines, mucins, and nitrogenous products and other glycoproteins (Fig. 2) [27–30]. It has

Table 1
Estimated number of head and neck cancer cases and deaths according to different sites in India & worldwide in both sexes in 2020 [2].

HNSCC subtypes	Annual Incidence		Annual Deaths	
	Worldwide	India	Worldwide	India
Lip & Oral Cavity (Mobile & other site of tongue)	377,713	135,929 **(36 %)**	177,757	75,290 **(42.3 %)**
Oropharynx (Base of the tongue)	98,412	20,617 **(21 %)**	48,143	12,703 **(26.4 %)**
Nasopharynx	133,354	5697 **(4.2 %)**	80,008	4148 **(5.2 %)**
Larynx	184,615	34,687 **(18.8 %)**	99,840	21,660 **(21.7 %)**
Hypopharynx	84,254	28,489 **(33.8 %)**	38,599	11,443 **(29.64 %)**
Salivary glands	53,583	7850 **(14.7 %)**	22,778	5127 **(22.5 %)**
Total cases	**931,931**	**232,819**	**467,125**	**130,371**

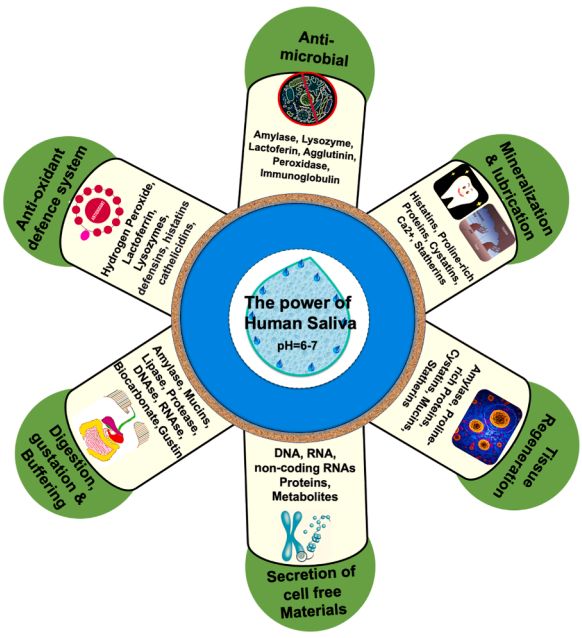

Fig. 2. Components and various physiological functions of human saliva.

various key roles including maintenance of electrolytes and ecological balance, pH of the oral cavity, cleansing and protection of the oral cavity, antimicrobial properties and aids in digestion etc. [31–33]. It also plays important roles during mastication, swallowing, digestion, taste perception and in speech [34]. Salivary proteins (mucins, lactoferrin, lysozyme, cystatins histatins, α-amylase and immunoglobulins) display good diagnostic potential in monitoring and detecting oral disease and oral cancers. Salivary mucins and amylases have anti-microbial activities within the oral cavity, increasing their retention in the dental pellicle, and/or protect proteins from proteolytic degradation through the formation of complexes [35]. So, the protective role and benefits including buffering, remineralization in the healthy oral mucosa, immune defense, digestion, lubrication, diagnostic purpose, and proteome analysis are fulfilled by saliva. In addition to the advantages of an easy, non-invasive, inexpensive, potential diagnostic and safe material, and it becomes a substitute for other biological fluids such as serum, blood, urine or smear in disease diagnosis and has significant potential for monitoring general health and systemic diseases with enormous translational values, and unprecedented prospects of clinical applications (Fig. 2). Due to the rapid development in the field of 'salivaomics' (genomics, transcriptomics, proteomics, metabolomics and miRNA analysis), salivary diagnostic may serve as a point-of-care tests for various oral and systemic diseases in near future.

Saliva, a simple non-invasive biological material useful for an early diagnosis of head & neck cancers

HNSCC is the 6th most prevalent cancer in the world [2,36], and number one cancer among Indian males. The abysmal overall survival and unfavorable outcome of HNSCC treatment due to diagnosis at the advanced stages (III-IV), lymph node metastasis and tumor recurrence [37]. Evidence suggests, early diagnosis and timely treatment of lesions, facilitate advance disease prevention, decrease tumor recurrence and metastasis, leading to improved overall survival of HNSCCs [38]. Currently, histopathological oral investigation is used as a standard method for HNSCC diagnosis, although it cannot fully reveal the dynamics of disease; however, liquid biopsy-based investigations are promising source for detection of molecular biomarkers for HNSCC patients [39,40]. Saliva-based diagnostic test is considered one of the best biofluids for HNSCCs diagnosis, as it is a non-invasive, safe, sensitive, affordable and painless collection for early diagnosis, prognosis and follow-up of HNSCC disease progression. Saliva contains various molecules such as DNA, RNA, miRNAs, proteins, cytokines, circulating tumor-derived cells and extracellular vehicles and has ability to reflect genomic, proteomic, transcriptomic, epigenomic, metabolomic, microbiomic and pathological alterations in head and neck region, therefore, saliva can be used as a potential source of liquid biopsy for early disease detection and could be used for biomarkers discovery for HNSCC patients [39–43]. Despite above potential advantages, use of saliva-based biomarker analysis and its clinical applicability is limited.

Salivary biomarkers in head and neck cancer

Identification of HPV specific biomarkers in saliva of HNSCC patients

Persistent infection with oncogenic human papillomaviruses (HPVs) are associated with anogenital (cervical, anal, vaginal, vulvar, and penile) cancers and a possible carcinogenetic factor for oral carcinoma at different sites (tongue, buccal mucosa, tonsil, larynx and oropharynx etc.) [44,45]. Growing epidemiological and molecular studies have shown strong association of HPV with a substantial proportion of HNSCCs such as oral, tongue, oropharynx, palate, lingual and tonsil cancer [46–51]. The prevalence of HPV infection in HNSCCs varies widely depending on the geographic region, HPV-DNA detection method employed, the demography, type of clinical sample used, and the anatomical site of the tumor. Globally, the prevalence of HPV infection in HNSCC has been recorded to be between 12−70 % [46, 52–55]. Surprisingly, the incidence of HPV-associated HNSCC is rapidly rising (42–70 %) in younger population mainly in developed countries [55,56]. High risk-HPV type 16 is the most predominant type and accounts for more than 90 % of HPV-linked HNSCCs [50,57–59]. Interestingly, HPV-driven HNSCCs are well-differentiated tumors and show better prognosis [4,45,58,59], while HPV^{-ve} HNSCCs exhibit poorly differentiated tumors and have worst prognosis and majority of them are tobacco users. Intriguingly, HPV^{+ve} oropharyngeal squamous cell carcinomas (OPSCCs), though also show favourable prognosis, 10–25 % of these patients experience disease recurrence within 2 years but majority patients manifest recurrence by 5 years or later [60]. HPV + OPSCCs also show a distinct mutational landscape [61,62] as compared to HPV^{-ve} tumors [59,63]. Therefore, a robust and effective diagnostic system should be in place to identify high and low-risk HPV^{+ve} OPSCC patients for effective treatment. Also, since HPV positivity drives sensitivity to radiotherapy, it is obvious that variations in HPV biology may cause differences in treatment response and outcome.

Despite increased prevalence and recurrence rate of HPV-associated HNSCCs (particularly oropharyngeal cancers and HPV-ve HNSCCs), there are currently no established biomarkers and no routine screening program is in place for early detection or prognosis of the disease. Considering the absence of screening and risk of late diagnosis of HNSCCs, it is an urgent need to search for specific and sensitive molecular biomarkers and implementation of screening methods for early diagnosis and effective treatment of HPV-associated HNSCCs [64].

Growing number of investigations have revealed that oncogenic-HPV-DNA can be examined in saliva of HPV-driven HNSCC patients which may help in improving disease detection, treatment outcome and monitoring [15,65–67]. HNSCCs lesions often shed cells with HPV-DNA/RNA into saliva, which can be used as a diagnostic biomarker for early detection [68,69]. Because of the close proximity and easy access to oral malignancies, saliva screening has received a lot of attention for early detection of HPV-driven HNSCCs [70]. Recently, it has been shown that occult HPV-driven oropharyngeal carcinomas (OPSCCs) can be investigated through saliva screening test [15]. Additionally, the identification of high-risk HPV (HR-HPV) E6 and E7 oncoproteins in saliva is gaining recognition as a promising diagnostic and prognostic biomarker, particularly for OPSCCs [71]. The non-invasive detection of HPV E6 and E7 mRNA in saliva has been

Table 2
Specific salivomic biomarkers in HNSCC.

Types of biomarkers	Role in HNSCC Diagnosis/Prognosis	Sensitivity (%)	Specificity (%)	References
Salivary transcriptomic markers				
EGFR	Oncogenic; Predicts treatment response	-	-	[90,118,119]
NOTCH1, PIK3CA, RAS	Oncogenic, Diagnostic marker	-	-	[78,90,94,100, 120]
IL-8 mRNA, IL-6 mRNA	Inflammation marker; HNC progression	99	90	[92,121-123]
P16INK4a mRNA, DAPK1	Tumor suppressor gene; Prognostic marker	94	87	[100,102,124]
Salivary proteomic markers				
IL-6, IL-8, TNF-alpha	Inflammation marker; Associated with HNSCC progression	89	78	[125,126]
EGFR	Implicated in HNC growth and progression	-	-	[92]
NF-κB	Inflammation marker; Prognostic marker	-	-	[127,128]
STAT3	Tumor progression; Prognostic marker	-	-	[128,129]
Salivary epigenetic markers				
miR-29a, miR-146a, miR-155	Epigenetic regulator; Prognostic marker	78	85	[117,130,131]
miR-21, miR-512, miR-10b, miR-412, miR-517b	Oncogenic miRNAs; Indicative of presence and recurrence of HNSCC	60	90	[39,114,132, 133]
miR-31, miR-193b, miR-let-7b	Tumor suppressor; Prognostic marker	80	65	[115,134,135]
DNA methylation of p16INK4a, TIMP-3, CCNA1, MGMT	Tumor suppressor gene inactivation; Diagnostic and prognostic potential	65	96	[72,100,136, 137]
Salivary genomic markers				
TP53 Mutation	Tumor suppressor gene; Prognostic marker	-	-	[75,79,138]
EGFR Amplification	Oncogene amplification; diagnostic marker	-	-	[139]
HPV DNA detection and E6, E7 Integration	HPV-associated HNC; Predicts prognosis	52.8	100	[140–142]
Salivary metabolomic markers				
Glutamine, pipecoline, Lactate	Tumor metabolism; Diagnostic marker	100	96	[143,144]
N-acetyl-l-phenylalanine, propionylcholine, sphinganine, phytosphingosine, and S-carboxymethyl-l-cysteine	Altered metabolism; Diagnostic	100	96.7	[145]
Glucose, carnitine valine, lactic acid	Altered metabolism; Diagnostic/Prognostic marker	86.5	82.4	[144,146,147]
Choline, alanine	Diagnostic marker	100	96	[144,148]

proposed as an early biomarker for HPV-positive HNSCC, as summarized in Table 2 [72,73]. In the primary HPV screening, 43.2 % cases were found positive for HR-HPV DNA and 81.4 % for p16 in saliva of OPSCCs patients with significantly a better prognosis compared to HPV^{-ve} patients. Studies have also shown that saliva-based assay is an effective and highly specific and reliable approach for HPV detection in $p16^{+ve}$ HNSCC tumours [66,74] (See table 2). These findings emphasize the unique role of saliva as a potential screening material for HPV-driven HNSCCs. However, salivary HPV detection has been observed to have varying degrees of sensitivity, specificity and prognosis in HNSCC patients [15,65–67]. As a result, salivary testing has not been received a widespread agreement on the utility of salivary screening as a standard biomarker analysis for early identification and monitoring of HPV-driven HNSCC. Further studies are needed to define a standard protocol reliable testing assays and guidelines for stringent testing and validation of results in using saliva for HNSCC patients which will help in making salivary HPV diagnostics a reality in future.

Tumor suppressor protein p53; a prognostic biomarker for HPV-linked head and neck cancer

The tumor suppressor protein p53 stands out as a crucial prognostic biomarker in HPV-linked head and neck squamous cell carcinoma (HNSCC). Frequently altered in HNSCC, p53's mutation patterns and interactions with HPV oncoproteins significantly influence cancer etiology and patient outcomes [59,63]. In HPV-positive HNSCC, p53 typically remains unmutated but is functionally inhibited by the HPV-E6 protein, leading to its inactivation/degradation [75]. This scenario contrasts with HPV-negative HNSCC, where p53 mutations are common, which often lead to stable expression of p53 indicating a poorer prognosis [76]. Wild-type p53 in HPV-positive cancer is associated with a favourable prognosis and enhanced survival rates [57,58], emphasizing the prognostic significance of p53.

The dependence of HPV-positive tumors on viral mechanisms to inactivate p53 suggests a vulnerability that could be exploited therapeutically to restore p53 function or hinder the HPV E6 protein, offering a tailored treatment strategy [77]. These insights have spurred interest in developing targeted therapies that capitalize on the differential p53 status between HPV-positive and negative HNSCCs [78]. Smith et al. (2010) proposed a biomarker panel, including HPV status, p16 expression, and p53, to improve survival predictions in HNSCC patients [79] (see Table 2). Therefore, p53's role extends beyond mere biomarker status, contributing to the refinement of therapeutic decisions [23]. The unique molecular pattern of p53 in the presence of HPV underscores its potential in precision oncology, highlighting the role of p53 protein as a major player in contemporary cancer research.

Identification of gene mutation in saliva of HNSCC patients

According to TCGA (The Cancer Genome Atlas) data, mutations in oncogenes, anti-oncogenes, or cell cycle regulating genes or DNA repair genes can cause variety of cancers including HNSCCs [80,81] .These mutations in several oncogenes or tumor suppressor genes have been linked to the development, progression and recurrence of multiple cancers. Emerging investigations have uncovered numerous genomic abnormalities associated with HNSCC patients who have history of tobacco smoking/chewing, alcohol consumption and HPV infection [23, 36,82]. It is well-known that, as HNSCC tumors grow, tumor cells and genetic material sheds into various body fluids such as urine, serum and saliva [68,83,84].

Therefore, using non-invasive body fluids often termed as liquid biopsy for analysing cancer-related gene mutations is one of the most promising methods for early identification of HNSCCs and other tumor biomarker candidates which will aid in a better understanding of the genetic background of these tumors (Fig. 3). Because of its easy, non-invasive, safe and simple collection procedure, saliva is becoming an emerging biofluid-specimen for early disease diagnosis, gene expression and mutation analysis. Currently, ≥100 salivary molecules such as DNA, RNA, non-coding RNA, exosomes, proteins and metabolites have been detected as potential molecular biomarkers for HNSCCs. Thus, it is

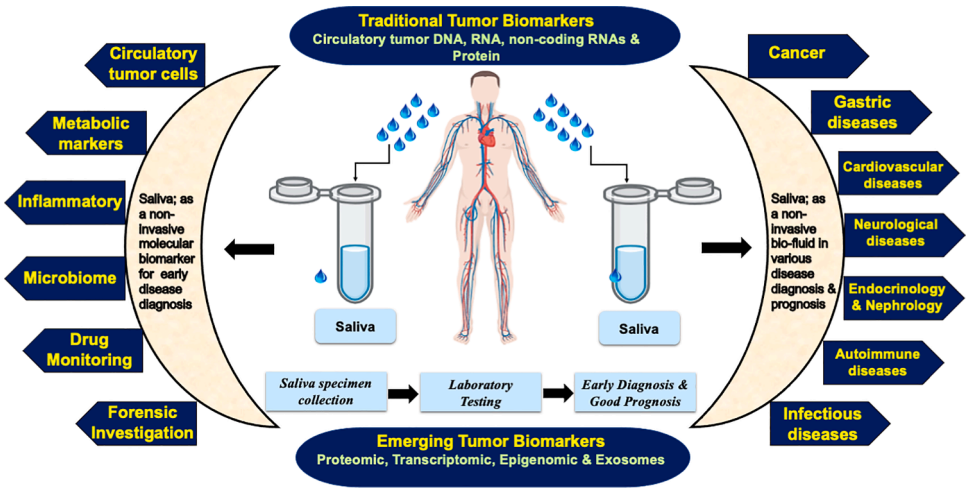

Fig. 3. Potentials of salivary diagnostic tests in different disease diagnosis and biomarker discovery.

practicable to identify differential mutational markers in saliva to diagnose and to know genetic profile of HNSCC tumors [16,17]. Earlier it has been shown that tumor-specific mutations are found in 71 % (5/7) saliva specimens collected from head and neck cancer patients. Lower expression of BRCA1 and BRCA2 genes was often associated with breast and ovarian cancer progression but mutation in BRCA pathway genes also contribute significantly to susceptibility of head and neck cancer [85]. HNSCC tumor cells and tumor-derived circulatory DNA (ctDNA) have been found in saliva of HNSCC patients [69,86]. Non-invasive oral rub and rinse (ORR) is an alternative technique for identification of gene mutation/genetic alterations for saliva-based early HNSCC detection [87]. Recently, Shanmugam et al. revealed that 93 % mutations were found in saliva of primary oral malignancies. Authors have also performed a multistep analytical validation and re-sequencing in 46 saliva samples and 88 % somatic mutations were analysed in these tumors indicating that saliva can be used for identification of somatic gene mutations in HNSCC patients [88]. Further, the higher expression of three salivary proteins, complement factor H (CFH), alpha-1-antitrypsin (SERPINA1) and fibrinogen alpha chain (FGA) were also associated with advanced grades of HNSCC tumors [89]. Current research highlights NOTCH1, EGFR and PIK3CA mutations within HNSCC tumors [78,90,91] and intriguingly in patient saliva, suggesting a potential for these mutations as non-invasive biomarkers for early detection and disease monitoring [92]. Notably, it has been reported that a significant link between salivary NOTCH1 mutations and tumor characteristics, underscoring its prognostic value [61,93,94]. Longitudinal liquid biopsy (saliva) analysis could help in identifying somatic mutations associated with HNSCC development [95]. In addition, a number of retrospective studies have shown that gene mutations are detectable in saliva of HNSCC patients and saliva-based identification of tumor-DNA showed better results compared to plasma-based detection preferably in early-stage tumors [68,84,88].

Potential of gene promoter methylation as salivary biomarkers in HNSCC

Recent advances in molecular biology have revealed that saliva harbors a wealth of biomarkers, including genetic and epigenetic signatures such as gene promoter methylation. Gene promoter methylation is a heritable and stable epigenetic modification involving the addition of methyl groups to specific regions of DNA, particularly in promoter regions of gene. Recent studies have identified epigenetic changes in HNSCC tumor tissues and saliva samples, providing a non-invasive approach in detecting and monitoring HNSCC [96]. The methylation-specific PCR and profiling methods have shown good associations with disease progression and clinical outcomes thus offering a highly sensitive, cost-effective, simple and time-efficient approach for detecting relatively low concentrations of methylated sequences in salivary samples, potentially aiding treatment decisions [16,97–99]. A quadruple-methylation marker panel comprising p16INK4a, RASSF1A, TIMP3, and PCQAP/MED15 tumor suppressor genes exhibited excellent diagnostic accuracy in the early detection of HNSCC. The panel achieved a remarkable sensitivity of 91.7 % and a specificity of 92.3 % in distinguishing HNSCC cases from healthy controls. Moreover, this panel demonstrated exceptional performance in detecting oropharyngeal cancer, achieving a remarkable sensitivity of 99.8 % and a specificity of 92.1 % [100]. The aberrant hypermethylation-induced inactivation of the MGMT promoter has been linked to a higher occurrence of GC > AT transition mutations in the TP53 gene and the KRAS oncogene, thereby contributing to HNSCC initiation and the progression [101]. A meta-analysis has provided compelling evidence regarding the association between salivary DNA promoter hypermethylation and the risk of HNSCC. The comprehensive analysis of available data from several studies revealed a significant and robust link between the methylation status of specific tumor-related genes such as p16, MGMT, DAPK, TIMP3 and RASSF1A exhibiting higher odds ratio (From 3.75 to 7.69, 95 % CI: 2.51–5.60 to 3.88–15.23) [102] and the risk of developing HNSCC (see Table 2). These studies highlight the clinical significance of salivary DNA promoter hypermethylation in early detection, risk assessment and better management of HNSCCs.

Identification of non-coding miRNAs in saliva of HNSCC patients

Small non-coding RNAs (ncRNAs) identification in saliva are emerging as new regulators of various biological functions which can help to diagnose their important roles in tumor initiation, progression and prognosis. These small ncRNA (piRNA, miRNAs, snoRNAs and lncRNAs etc.) molecules are consisting ~98 % of all transcriptional output in humans and are very stable in variety of body fluids including saliva and less susceptible to degradation by RNases, suggesting their potential role in biomarker discovery. Therefore, NcRNAs are appearing as very attractive, ideal and suitable salivary molecules for the discovery of new diagnostic makers and/or therapeutic target(s) for HNSCCs (Fig. 3) [103,104]. miRNA profiling was performed in saliva for oral cancer screening and detection [105–107]. Several studies have shown the potential role of salivary ncRNAs for identification of various human diseases including head and neck cancer, breast cancer, pancreatic cancer, cervical cancer, lung cancer and ovarian cancer [42,108–112]. In a meta-analysis, the combined sensitivity of salivary miRNA for the diagnosis of HNSCC was found to be 69.7 % with a 95 % confidence interval (CI) ranging from 64.4 % to 74.4 %. The specificity was

determined to be 86.8 %, with a 95 % CI ranging from 81.1 % to 91.0 %. [113]. The higher expression profiles of miR-146a, miR-155, miR-31, miR-21, miR-10b, miR-375 in saliva positively correlated with the severity of diseases in oropharyngeal and head neck cancers, underscoring their prognostic value (see Table 2) [114–117]. Hence, the development of a nc-RNAs-based salivary test, could have a significant importance for clinical practice for early diagnosis of HNSCC patients.

Diagnostic and prognostic role of salivary exosomes and exosomal-miRNA for early HNSCC detection and drug delivery

Human saliva has many biological functions as it contains various proteins, hormones, DNA, RNA, miRNAs, exosomes and exosomal miRNAs [28–30]. Exosomes are small, lipid bilayer-enclosed extracellular micro-vehicles (EVs) derived from endosomes with a diameter of 50–150 nm that can detected in a wide range of body fluids including saliva and contain a variety of substances, including proteins, lipids, and nucleic acids (DNA, mRNA, miRNAs, long non-coding RNAs, circular RNAs, piRNAs and ribosomal RNAs) [149,150]. Exosomal miRNAs have longer-life spans because they are produced from multivesicular bodies (MVBs) that are protected from RNases [29]. By fusing MVBs with the plasma membrane, exosomes are released into the extracellular space and circulated in the all-body fluids such as blood, serum, plasma, urine and saliva. Exosomes transport miRNAs and proteins and play an important role in signal transmission for intercellular communication [151,152]. As a result of their prospective involvement as novel cancer biomarkers, diagnosis and therapeutics, salivary exosomes have gained more attention now days. Thus. salivary exosomes, being a readily available bodily fluid, could be a useful indicator for evaluation of various disease pathogenesis including cancer [153].

Emerging studies have shown that salivary exosomes and derived miRNAs as a non-invasive biomarker for early detection and diagnosis of various cancer including HNSCCs [154–156]. miRNAs that found in salivary lipoprotein vesicles (called exosomes) can be used as reliable diagnostic biomarkers for pathogenic diseases including variety of cancers [103,157,158]. Recently, it has been shown that exosomes have been linked to HNSCC pathogenesis, and enhance HNSCC proliferation, invasion and metastasis by transferring their contents to target cells. EVs derived from cancer cells may promote metastatic niche formation by creating a favourable micro-environment for cancer cell. Further studies have also shown that cancer-derived EVs are associated with disease invasion and chemo-radio resistance [159,160]. Serum exosomal miR-21 expression in HNSCC patients found higher than in chronic hepatitis patients and healthy subjects [158]. Current research pointed that salivary exosomal miRNAs can serve as potential novel non-invasive biomarkers for early detection of HNSCCs. According to Peacock et al. EVs-derived miRNA cargo is associated with HPV status in HNSCCs [161]. Further, alcohol use has also been linked to the circulating exosomes formation. The authors have also shown that following binge drinking, the expression of miR-122 was dramatically increased in the sera exosomes of healthy individuals [162]. Recently, it has also been identified that a significant higher expression of miR-24–3p in salivary exosomes was associated with proliferation in HNSCC patients compared to normal individuals [156]. These evidences have shown that salivary exosomes and derived exosomal miRNAs can be employed as non-invasive, specific and sensitive diagnostic and/or therapeutic biomarkers for early detection of various cancers more especially HNSCCs (Fig. 3).

Salivary cell-free DNA or circulatory tumor DNA: a promising approach for non-invasive HNC diagnosis and monitoring

Liquid biopsies using bodily fluids offer a promising, non-invasive approach for cancer diagnosis, prognosis, therapy assessment, and identifying gene mutations and molecular targets. In normal cell turnover and specific disease contexts, dying cells release DNA/RNA and cellular debris into body fluids. While healthy individuals efficiently clear these materials through phagocytosis, cancer patients experience an impairment in this process leading to accumulation of cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA) in tissue microenvironments and biological fluids [163]. Therefore, cancer patients exhibit elevated level of cfDNA in their body fluids [84,164]. CtDNA, originating from cancer cells can be distinguished from cfDNA released by non-cancer cells by characteristics such as biofluid concentration, somatic mutations, and size.

One notable component of cfDNA is salivary tumor DNA (stDNA), which has garnered significant attention in non-invasive diagnostics. stDNA comprises DNA fragments released into oral fluid from various cells in the mouth such as, oral epithelial cells, immune cells and cells from distant locations of the body. stDNA can effectively indicate the presence of malignant lesions in the oral cavity, pharynx, and other head and neck regions, making it a powerful tool for screening and monitoring. The advantages of using salivary cfDNA is its ease of collection, providing an easy alternative to blood or tissue biopsies. Further, it serves as a dynamic and accessible liquid biopsy, capable of reflecting the real-time changes in health status of an individual and hence salivary cfDNA holds the promise of revolutionizing disease detection, monitoring, and personalized medicine [165,166]. CtDNA was detected in 100 % of early-stage HNC patients and in 95 % of late-stage patients. There is a consistent enrichment of stDNA in the saliva of OSCC patients, highlighting its specificity and potential for OSCC diagnosis and monitoring [167]. Furthermore, ctDNA was found in 100 % of post-surgical patients who later experienced clinical recurrence while it was absent in those without recurrence. The analysis of genetic alterations associated with HNC in ctDNA from both saliva and tissue samples also revealed very good correlations. Moreover, epigenetic alterations, particularly gene promoter methylation, can be investigated using salivary ctDNA. This underscores the potential of salivary ctDNA methylation as a highly promising biomarker for HNC diagnosis, prognosis, and follow-up [168].

Salivary mitochondrial DNA and cell-free mitochondrial DNA: promising biomarker for head and neck cancer diagnosis and prognosis

Mitochondrial DNA (mtDNA) has been identified as a promising non-invasive biomarker for HNSCC, notable for its maternal inheritance and distinct location within mitochondria. The 16.5-kb mtDNA, which encodes vital components for cellular respiration, is increasingly examined for mutations that may serve as indicators of cancer [169,170]. The 16.5-kb mtDNA encodes essential components for cellular respiration and is a key target for mutation detection in cancer patients [171].

Mitochondrial DNA (mtDNA) has been identified as a promising non-invasive biomarker for head and neck squamous cell carcinoma (HNSCC), notable for its maternal inheritance and distinct location within mitochondria. The 16.5-kb mtDNA, which encodes vital components for cellular respiration, is increasingly examined for mutations that may serve as indicators of cancer [169–171]. Salivary DNA analysis offers a patient-friendly diagnostic alternative, providing a window into cellular alterations through mtDNA present in epithelial cells and leukocytes [172]. The displacement-loop (D-loop) region of mtDNA, in particular, has been associated with HNSCC, with mutation frequencies that enhance diagnostic accuracy over cell-free nuclear DNA (cfDNA) [173–175].

In parallel, the advent of cell-free mitochondrial DNA (cf-mtDNA) analysis has broadened the biomarker landscape for HNSCC. Characterized by mitochondrial fragments in the cell's extracellular milieu, cf-mtDNA has shown superior diagnostic specificity in various malignancies, underscoring its potential in early cancer detection and disease monitoring [172,176]. The high sensitivity (84 %) and specificity (100 %) observed in recent studies establish cf-mtDNA as a valuable diagnostic tool, with a notable correlation to HNSCC [177]. Notably, individuals with HNC exhibited higher levels of cf-mtDNA (29,103,

476.15 genomic equivalent/mL) compared to normal controls (9189, 312.54 genomic equivalent/mL) [177]. These findings highlight the strong correlation between elevated cf-mtDNA content and risk factors such as smoking, tobacco chewing, and alcohol consumption, positioning it as a promising diagnostic biomarker characterized by minimal invasiveness, high specificity, and sensitivity [177–180].

The convergence of findings regarding salivary mtDNA and cf-mtDNA fortifies the position of mitochondrial biomarkers in the minimally invasive detection and management of head and neck cancer, epitomizing a shift towards more personalized and patient-centered care.

Salivary cytokines and antibodies as potential biomarkers for head and neck cancer

The complex pathophysiology of HNSCC involves complex interactions between tumor cells and the host immune system, resulting in altered immune molecules cytokine and antibody expression profiles and immune-modulation leading to the tumor initiation, growth, invasion and metastasis [181]. The complexity of HNSCC pathophysiology necessitates novel approaches for early detection and personalized treatment strategy. The non-invasive nature of saliva collection and its direct contact with the oral and oropharyngeal mucosa, where early cancerous changes often occur, make it an attractive medium for analysis of inflammatory and immune biomarker(s). Salivary cytokines and antibodies (IgA, IgG and infection-specific IgM) have emerged as promising biomarkers for the early diagnosis, prognosis and monitoring of HNSCC due to their pivotal roles in mediating immune responses and inflammation [182]. This approach capitalizes on the unique composition of saliva, which contains a plethora of molecules reflective of systemic and local physiological changes, including the intricate interplay between cancer cells and the immune system. Emerging studies have shown that altered cytokine profile including interleukins (IL-6, IL-8), tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β), VEGF in the saliva of HNSCC patients was correlated with the severity of oral lesions, treatment response and overall survival [122,123,128,183,184]. IL-1, IL-6, IL-8, and TNF-α were involved in HNC cell proliferation and survival leading to up-regulation of NF-kB, STAT MAPK/ERK pathways [126] (see Table 2). In a study conducted by Deepthi et al., the salivary levels of TNF-α were examined in a cohort comprising 30 patients diagnosed with OSCC, 30 oral-leukoplakia patients, and 30 healthy controls [185]. The results revealed notable differences in TNF-α concentrations among the groups. Patients afflicted with oral cancer exhibited a significantly higher level (43.75 pg/ml) of salivary TNF-α concentration compared to those with leukoplakia (21.825 pg/ml). These findings underscore the potential of salivary TNF-α as a biomarker for distinguishing between precancerous verses cancerous lesions [186]. Polz-Dacewicz et al. conducted an analysis of IL-10, TNF-α, TGF-β, and VEGF levels both in saliva and serum samples collected from a cohort of 78 oropharyngeal cancer patients. Strikingly, the concentrations of all four cytokines were found to be significantly higher in saliva when compared to serum levels. Notably, the study also delved into the presence of HPV and Epstein-Barr virus (EBV) DNA in tumor tissues, revealing that patients with detectable EBV DNA had the highest IL-10 concentrations, while those with detectable HPV DNA displayed the highest TGF-β concentrations. Furthermore, the levels of TNF-α and VEGF were found to be correlated with the histological tumor grade and tumor size [187], underscoring the potential of these cytokines as valuable indicators in assessing oral disease severity and progression [188]. Salivary antibodies mainly secretory IgA, IgG and oral pathogen-specific IgM reliably reflect both mucosal and systemic immunity of the patient [89,189].

Prognostic role of pan-immune-inflammation values in head and neck cancer

The emerging role of pan-immune-inflammation values in head and neck cancer prognosis is gaining acceptance. Increasingly, evidence supports the use of compound scores based on blood counts as valuable prognostic biomarkers in cancer, offering insights into the degree of uncontrolled inflammation within the tumor microenvironment [190,191]. While various markers have been developed for this purpose, the pan-immune-inflammation value (PIV), introduced by Fuca et al. in 2020, stands out. PIV, takes into account blood neutrophils, monocytes, platelets, and lymphocytes, has exhibited superior predictive capabilities for survival outcomes, surpassing other markers in patients with metastatic colorectal cancer [192,193]. PIV has demonstrated promise in predicting outcomes across various solid tumors, including HNC as a reliable prognostic marker [192–195]. A recent meta-analysis involving 30 studies in 8799 HNC patients has highlighted the potential of pre-treatment Pan-Immune-Inflammation Value (PIV) as a non-invasive and effective prognostic biomarker. This study unveiled a significant association between higher PIV and poorer overall survival (HR = 2.07; 95 % CI: 1.77–2.41) and progression-free survival (HR = 1.83; 95 % CI: 1.37–2.45).

Multivariate analyses, accounting for various factors, consistently reaffirmed the independent prognostic importance of PIV for both overall and distant metastasis-free survival in OSCC patients [195]. Elevated PIV levels were found to correlate with specific clinicopathological factors [196]. It is strongly suggested that PIV could serve as a minimally invasive prognostic biomarker in various cancers including HNCs.

Prognostic value of PD-L1 expression in head and neck cancer

Programmed death-ligand 1 (PD-L1), an immunoreceptor protein pivotal for cancer immune evasion, has been recently consideration as an important biomarker for treatment outcome in cancer. Its expression in tumor cells and associated immune cells impedes T-cell-mediated immune responses, fostering cancer progression. Recently estimating the potential of PD-L1 level in saliva in addition to tumor tissues, highlights its significance in head and neck cancer (HNC) therapy [197–199]. Elevated salivary PD-L1 has been correlated with its tumor tissue expression, establishing its prospective role in diagnosis, prognosis, and therapy [200–202]. Studies suggest that while tissue biopsy is a gold standard for PD-L1 assessment, non-invasive salivary analyses might represent a transformative alternative [203]. With the growing importance of successful PD-1/PD-L1 pathway-focused immunotherapies in cancer [204], a comprehensive evaluation of PD-L1 expression in both tissue and saliva becomes imperative.

Sarcopenia as a prognostic marker in elderly head and neck cancer patients

Sarcopenia, characterized by the progressive loss of skeletal muscle mass and strength, is commonly associated with aging and chronic conditions of elderly person [205]. Emerging research highlights its significance as a prognostic marker in various cancers, especially HNC [206]. Within HNC contexts, sarcopenia often parallels the cancer diagnosis, likely due to tumor-induced metabolic changes and decreased nutrient intake from tumor-related symptoms [207]. Sarcopenia in HNC patients has been linked with reduced treatment tolerance, heightened postoperative risks, and poorer survival outcomes [208,209]. Studies by Grossberg et al. (2016) and Stone et al. (2019) further accentuate its negative impact on survival, emphasizing its value as a potential biomarker for prognosis and treatment outcomes in HNC [210,211]. Intriguingly, recent studies are exploring saliva as a non-invasive medium to detect sarcopenia [212]. Studies suggest possible correlations between salivary proteins, metabolites, and sarcopenia-related

conditions [213]. Though this avenue promises easy andsearly detection and monitoring approaches, extensive clinical validation required before introduction in routine clinical practice.

Salivary microbiome signatures in HNSCC diagnosis and prognosis

Oral-microbiome is a highly complex group of microbes that play key roles in a variety of physiological processes and maintaining oral health. The oral microenvironment at different sites in the mouth cavity has different microbial compositions which is highly regulated by complex signaling, hosts, viral and external environmental factors. Disruption in oral microbiota through tobacco smoking, alcohol consumption, poor immunity, infections with pathogenic microorganisms may lead to development of oral diseases including HNSCC [214]. The oral-microbiota have now emerged as potential biomarkers for oral cancer progression [215]. Growing number of reports have shown that salivary microbiome was found in HNSCC patients and revealed its potential application as a diagnostic tool for oral cancer screening [216, 217]. It is also reported that salivary microbiome can reflect pathobiology and stage of the oral disease [218,219]. Thus, identification of changes in salivary microbiome and associated biomarkers could be a useful tool for early screening of HNSCC patients and provide new insight with respect to pathobiology of disease and therapeutic interventions.

Salivary metabolites: a next-generation biomarker for early detection of head and neck cancer

Saliva, a biofluid rich in molecular constituents, has gained prominence as a diagnostic medium due to its ease of collection and its potential to reflect systemic changes associated with disease status including development of head and neck and other cancers. Metabolomics, a high-throughput analytical technique, enables the comprehensive profiling of small molecules in biological samples, offering valuable insights into metabolic alterations linked to disease states. Salivary metabolomics holds great promise as a non-invasive and accessible method for systemic early detection and diagnosis of cancer. Additionally, Salivary metabolomics offer insights into the underlying metabolic processes involved in cancer progression, paving the way for developing personalized treatment strategies. Emerging studies have identified specific metabolite profiles associated with HNSCC, allowing discrimination of cancer patients from healthy individuals and those with benign lesions. These metabolites, including alterations in amino acid, lipid, and carbohydrate metabolism, serve as potential biomarkers for early detection and monitoring of HNSCC [220]. Further, salivary metabolites also hold high prognostic value, helping predict oral cancer progression and clinical outcomes following treatment [221]. Earlier study by Wang Q et al. (2014), 14 salivary metabolites were identified as potential biomarkers, showing 8 upregulated and 6 downregulated metabolites in HNSCC patients when compared to controls [145]. Notably, a combination of 5 salivary biomarkers (N-acetyl-l-phenylalanine, propionylcholine, sphinganine, phytosphingosine, and S-carboxymethyl-l-cysteine) demonstrated remarkable accuracy (AUC = 0.997), sensitivity (100 %), and specificity (96.7 %) in effectively distinguishing patients with stage I-II HNSCC from the healthy controls [145] (see Table 2). Furthermore, Wei et al., (2011) utilized UPLC-QTOFMS analysis to pinpoint a distinctive panel of salivary metabolites (valine, lactic acid, n-eicosanoic acid, γ-aminobutyric acid, and phenylalanine) within 37 OSCC patient samples and distinguishing OSCC from their healthy counterparts with a sensitivity of 86.5 % and specificity of 82.4 %. Notably, lactic acid and valine showed significant elevation in OSCC compared to oral leucoplakia [144]. In parallel studies, two groups investigators conducted salivary metabolomic profiling in oral cancer patients. Both groups consistently identified multiple metabolites, including cadaverine, glutamic acid, pyrrolinehydrocarboxylic acid, threonine, choline, beta-alanine, carnitine, piperidine, tryptophan, taurine, glutamine, leucine plus isoleucine, alanine, pipecolic acid, valine, and histidine, which were significantly elevated in the saliva and tumor tissues of patients compared to controls [222]. While Sugimoto's group showed taurine and piperidine as key oral cancer-specific markers ($p < 0.05$) within a pool of 69 OSCC saliva samples, Ishikawa et al. reported a substantial fold change value for kynurenine (FC = 38.1, $p < 0.0001$), a metabolite associated with reactive oxygen species-mediated stress in tumor samples from OSCC patients. Together these findings underscore the potentials of salivary metabolites to mirror the changes in tumor tissues, offering promise for the diagnosis and prognosis of HNSCC [221–223].

Revolutionizing cancer diagnosis: liquid biopsy vs. traditional tissue biopsy

Traditional tissue biopsy collection by invasive methods followed by histopathological diagnosis have been the gold standard procedures for diagnosis and characterization of various diseases, especially cancer [224,225]. Though these techniques have undoubtedly saved lives, they come with several limitations and drawbacks. Liquid biopsy mirrors diagnostic accuracy of tissue biopsy and provides non-invasive, real--time insights; a revolutionary approach that offers promise in overcoming challenges and difficulties of tissue biopsy (Table 3).

Developing novel testing platforms for saliva-based diagnostics: bridging the gap for reliable, consistent and faster results

The field of saliva-based diagnostics holds tremendous potential for non-invasive disease detection and monitoring. However, one significant challenge is the lack of standardized chip design, ELISA (Enzyme-Linked Immunosorbent Assay) and sensor design for the microfluidic platforms used in these diagnostics. To overcome this challenge and unlock the full potential of saliva-based testing, several suggestions and proposals can be considered:

ELISA-based saliva test; a promising non-invasive approach

The utilization of ELISA-based saliva tests represents a promising approach in the realm of diagnostic medicine. This method harnesses the power of Enzyme-Linked Immunosorbent Assay (ELISA) technology to detect a wide range of biomolecules in saliva, providing valuable insights into various health conditions. ELISA-based saliva tests have been explored in the diagnosis of numerous diseases, including infectious diseases and cancer [119]. Saliva contains a wealth of biomarkers, including nucleic acid, proteins, cytokines and antibodies, reflecting both local and systemic changes associated with HNSCC. ELISA, a highly sensitive and specific immunoassay, can detect and quantify these biomarkers in saliva samples [108,226]. Several key biomarkers, such as salivary proteins (e.g., matrix metalloproteinases, cytokeratins and interleukins) and cancer-associated autoantibodies, have demonstrated promising diagnostic potential [28,227]. Utilizing saliva-based ELISA not only offers early detection capabilities but also allows for disease monitoring and assessment of treatment efficacy. Emerging technologies such as microfluidics and multiplexed ELISA platforms enhances sensitivity and specificity. Offers potential for the creation of more precise and accessible diagnostic tools for patient care.

Saliva-based sensor for the diagnosis of HNSCC

The rapidly advancing field of biosensing and point-of-care diagnostics hold immense potential in advancing early detection of HNSCC. An innovative approach within this domain involves saliva-based sensors for HNSCC diagnosis [228]. These sensors capitalize on distinctive biomolecular patterns present in saliva to detect specific HNSCC markers (DNA, RNA and protein etc.), offering a sensitive, non-invasive and readily accessible method for early detection and

Table 3
The characteristics on which standard tissue biopsy-based histopathological diagnosis can be differentiated from those of liquid biopsy.

Characteristics	Tissue Biopsy	Liquid Biopsy
Invasiveness	Tissue biopsies are invasive, time consuming and cumber some risky procedures that may cause pain, discomfort and complications for patients.	Liquid biopsies are is minimally invasive or non-invasive and often require a simple blood draw or saliva or urine collection with minimal or no discomfort or risks to patients.
Sample Collection	Requires a specific target tissue sample using invasive procedure which may not always be feasible	Can be easily collected and repeated over time, reducing sampling errors
Timing of Procedure	Usually performed after the suspicion of a specific disease/pathologic condition.	Can be done at various stages, including early detection, during and following treatment.
Sampling Bias	Tissue biopsies provide a snapshot of a specific area within the tumor, which may not fully represent the entire tumor's genetic and molecular heterogeneity. This sampling bias can lead to misdiagnosis or an incomplete understanding of the disease biology/pathology. Self-sampling is not possible.	Reduced sampling bias. Since liquid biopsies capture genetic material or cells circulating throughout the body, they provide a more comprehensive and systemic view of tumor heterogeneity. Self-sampling can easily be done.
Real-Time Monitoring	Tissue biopsies offer a static view of the disease, capturing its status at a single point in time. They are ill-suited for monitoring dynamic changes, such as disease progression, treatment response, prognosis and the emergence of resistance or mutations over time.	Liquid biopsy enables real-time monitoring of cancer progression, treatment response, and the emergence of drug resistance, allowing for timely strategy for disease management and patient care.
Turnaround Time	Longer turnaround time due to time consuming procedure for sample collection, processing and histopathological analysis.	Generally faster results, which can be crucial for treatment decisions.
Risk to Patients	In addition to invasiveness, tissue biopsies carry risks such as bleeding, infection, damage to adjacent healthy tissue and possibility of mixing and spread of cancer cells through systemic circulation. These risks can be particularly of concern for patients with certain medical conditions like cancer.	Liquid biopsy procedures are generally safer and carry fewer or no risks compared to tissue biopsies, making them more accessible to a wider range of patients.
Tumor Heterogeneity	Limited ability to capture intra-tumoral heterogeneity.	Allows better sampling for evaluating tumor heterogeneity, providing a more comprehensive view
Detection of Minimal Residual Disease	Limited detection of minimal residual disease (MRD).	Improved sensitivity in MRD detection.
Limited Accessibility	Some tumors may be located in anatomically challenging or risky areas, making it difficult or dangerous to perform a tissue biopsy. This limitation can delay diagnosis and treatment.	Accessible for all patient population, even in remote areas as it is only a body fluid.
Delayed Diagnosis	The invasive nature and longer time required for tissue biopsy collection and processing can often lead to delays in diagnosis and treatment initiation, which can be critical in rapidly progressing diseases such as cancer.	Due to non-invasiveness and easy collection procedure, early diagnosis and treatment is possible.
Cost	Can be costly due to the need for surgical procedures and histopathological analysis	Generally, more cost-effective, especially in long-term disease monitoring
Man Power	Requires more no of well trained and medical specialist personnel to carry out surgical intervention and histopathological processing and diagnosis.	A single person can collect several samples and carry out analysis.
Handling of specimens	Handling of tissue sample is not very safe and not easy to store, transport, more expensive and time consuming.	Handling of saliva sample is safer as it is easy to transport, store, less expensive and less time require for both patients and clinicians.
Application	Predominantly used for definitive diagnosis and initial staging/grading.	Used for initial diagnosis, monitoring, treatment response assessment, and relapse detection.

monitoring. Recent studies by Chaiyarit et al. (2018) and Guerrero-Preston et al. (2019) have underscored the feasibility and accuracy of saliva-based sensors in HNSCC diagnosis [97,229]. The non-invasive nature of saliva collection enhances the appeal of this technology for routine screenings and patient monitoring, potentially elevating early detection rates and patient outcomes. Therefore, the development of saliva-based sensors has the potential to significantly advance HNSCC diagnosis by offering a rapid, cost-effective, and patient-friendly diagnostic tool.

Saliva-based PCR and qRT-PCR in HNSCC diagnosis

Saliva-based Polymerase Chain Reaction (PCR) and Quantitative Reverse Transcription PCR (qRT-PCR) have emerged as promising diagnostic tools for the early detection and monitoring of HNSCC. They have also proven effective in detecting various pathogens, including HPV, EBV, HSV-1 and SARS-CoV-2 [74,230-232]. These non-invasive techniques harness the precision of molecular biology to identify specific genetic markers associated with HNSCC in saliva samples, offering distinct advantages for early detection. In particular, RNA biomarkers like miRNAs and mRNAs exhibit distinct differential expression patterns in HNSCC patients [39,233].

Open-source designs

Promote open-source chip designs to foster innovation and knowledge sharing within the scientific community. By making designs freely accessible, researchers can build upon existing knowledge and contribute to the refinement of standardized chip designs.

Regulatory engagement

Engage with regulatory bodies to establish guidelines for microfluidic chip approval and adoption in clinical practice. Collaborate with agencies to develop a regulatory framework that supports standardized designs while ensuring safety and efficacy.

Funding and grants

Governments and private institutions should allocate funding and grants specifically for research and development in standardized chip designs for saliva-based diagnostics. These financial incentives can encourage researchers and companies to invest in creating reliable and cost-effective microfluidic platforms.

Industry collaboration

Foster collaboration between academia and industry to bridge the gap between research and commercialization. Industry partners can provide resources, manufacturing capabilities, and market access to accelerate the adoption of standardized chip designs.

Education and training

Develop educational programs and training initiatives to equip researchers and engineers with the skills and knowledge needed to design and fabricate standardized microfluidic chips. These programs can help cultivate a skilled workforce in the field.

Continuous improvement

Recognize that the field of microfluidics is dynamic, and technology evolves rapidly. Encourage ongoing research and development to continually refine and improve standardized chip designs as new technologies and insights emerge.

By implementing these suggestions and proposals, the field of saliva-based diagnostics can make significant strides toward overcoming the lack of standardized chip design and sensitive sensor platforms. Standardization not only enhances the reliability and accuracy of diagnostics but also paves the way for the widespread adoption of non-invasive, saliva-based testing in clinical practice, ultimately benefiting patients and healthcare systems and the society/humanity at large.

Collaborative imperatives to advance the clinical translation of salivary microfludic technology

Unlocking the potential of saliva-based diagnostics hinges on several key actions. First, we must establish standardized practices and enhance communication among academic research groups. This entails creating consistent and safe methods for saliva collection, processing, and storage to ensure the reliability of results across different studies and platforms. Additionally, fostering collaboration between clinicians and academic research teams is essential for expediting the discovery and validation of robust salivary biomarkers, addressing challenges related to biomarker variability and enhancing the clinical utility of saliva-based diagnostics.

Furthermore, the advancement of microfluidic technologies for clinical translation demands interdisciplinary collaboration. These technologies offer the promise of high-throughput, point-of-care saliva testing, but their development and optimization require engineers, biologists, and clinicians to collaborate closely. Overcoming issues like sensitivity, specificity, cost-effectiveness, and scalability in microfluidic platforms is crucial for realizing their full potential in precise and efficient disease diagnosis and monitoring within clinical settings. Identifying gaps in integrating parameters for broader applications in saliva-based biomarkers and addressing these challenges are essential for advancing personalized medicine and non-invasive diagnostics. This mission underscores the need for sustained long-term investment and multidisciplinary collaboration. To tackle these challenges effectively, we can focus on several essential areas:

Collaborative research initiatives

Encourage collaborative efforts among researchers, engineers, and clinicians from academia, industry, and healthcare institutions. Establish multidisciplinary teams dedicated to developing standardized chip designs. These teams can leverage their collective expertise to create versatile and reliable microfluidic platforms.

Establish guidelines and consortia

Formulate comprehensive guidelines and standards for microfluidic chip design in saliva-based diagnostics. Collaborative consortia involving experts in microfluidics, biotechnology, and clinical research can work together to define these guidelines. Such standards can help ensure consistency and reliability across different platforms.

Standardization of protocols

Collaborative efforts to establish consensus protocols are crucial for robust and reproducible liquid biopsy outcomes and addressing issues related to sample collection, processing, and data analysis [234,235].

Multimodal data integration and interpretation

Integrating diverse parameters from saliva, including genomics, transcriptomics, proteomics, metabolomics, microbiome and clinical data, remains a challenge. Advanced analytical techniques and artificial intelligence-based machine learning models needs be developed collectively to extract meaningful clinical insights from multi-dimensional datasets [236,237].

Biomarker discovery and validation

The non-invasive biomarkers identification and their validation for specific diseases demand extensive collaboration between clinicians, researchers, bioinformaticians, data analysts and biologists. Multidisciplinary teams can accelerate the discovery of reliable salivary biomarkers and ensure their clinical validity and utility [225].

Clinical validation

Large-scale, multi-centric clinical trials involving various stakeholders including healthcare institutions, research organizations, academic institutions, pharmaceutical companies, and regulatory bodies are essential for establishing the clinical utility of saliva-based diagnostics [238,239].

Ethical considerations

Collaboration between policymakers, ethicists, and researchers is essential to address ethical issues, such as patient consent, patient safety, data privacy, and clinical implementation in evolving and utilizing landscape of saliva-based diagnostics [240].

Cost-effectiveness and accessibility

Collaborative efforts between healthcare providers and technology developers are required to optimize cost-effectiveness and accessibility of saliva-based tests, making them accessible to a broader patient population [241].

In order to overcome these challenges, it is important to adopt a multifaceted approach that includes sustained long-term investment from both the public and private sectors, encouraging multidisciplinary collaboration through interdisciplinary research consortia, and development of open-access databases and resources to facilitate data sharing and accelerate progress in better diagnosis and treatment of various diseases including head and neck cancers.

Use of saliva in HPV vaccination monitoring

The primary steps that can help to reach the objective of eliminating HPV not just for cervical cancer but also in HPV-induced HNSCCs through HPV vaccination and regular HPV screening monitoring. The introduction of HPV vaccination program has significantly reduced the incidence of cervical cancer in several developed countries [242,243]. Despite many countries have effectively introduced HPV vaccination, there are several countries still lack surveillance, monitoring and even introduction of HPV vaccine. Current HPV vaccines have shown promising protection against HPV-associated cancers in both males and females [244].

Currently, there are six HPV prophylactic vaccines have been approved by US-FDA (Food and Drug Administration) for prevention

and control of HPV-related diseases: Cervarix; a 2 dose bivalent vaccine only for girls and boys aged 9–14 years for females against HPV16 and HPV18, while Gardasil; a tetravalent 2 dose vaccine for both females and males aged 9–13 years against HPV 6, 11, 16, and 18 and also a Non-avalent HPV vaccine (Gardasil-9) recommended for both girls and boys for protection against HPV types 6, 11,16, 18, 31, 33, 45, 52, 58 [245, 246]. Recently, another quadrivalent HPV vaccine Cervavac; a single or 2 dose schedule for boys and girls aged 9–13 years. This is a highly cost-effective vaccine being introduced in India with the approval of Drug Controller General of India (DCGI) and also approved by US-FDA [247]. There are still two more US-FDA approved 2-dose bivalent vaccines, Cecolin and Walrinvax licensed for girls of 9–14 years of age. These vaccines are administered intramuscularly in the deltoid with a standard dose of 0.5 mL and found to be safe, efficacious and tolerable and recommended for use to get protection from HPV-associated cervical, anogenital, oral, and oropharyngeal cancers [248]. Following the introduction of HPV vaccination, it is critical to evaluate a vaccine's immunogenicity, efficacy, safety in reducing the HPV-associated diseases, and long-lasting population safety. Current evidences support that saliva-based screening of HR-HPVs in younger generation may help in generating awareness about presence of HPV infection and its prevention by vaccination [249–251]. As HPV infection can be detected in saliva, this alternative non-invasive HPV diagnosis approach for HPV vaccine monitoring is becoming more attractive and acceptable as it is less tedious, safe and relatively coast-effective. Thus, it may support the implementation of vaccine monitoring programmes in low-resource settings, as well as reaching a larger population. These data highlight that National HPV Immunization Programme for both girls and boys and HPV awareness can be possible with non-invasive method of screening using saliva and urine for patients with HNSCCs and cervical cancer respectably in developing countries.

Concluding remarks

Saliva as a liquid biopsy remains a goldmine for discovering various biochemical, immunological, genetical and molecular biomarkers including numerous high-throughput markers in genomics, proteomics, epigenomics, microbiomics and metabolomics in HNSCCs and other diseases in humans. However, due to sensitivity and specificity, as well as technological constraints and expenses, the most of them have remained in the laboratory and have not been reached to clinics. Still there exists several challenges in salivary testing: 1). Although saliva samples represent a promising substitute to oral tissue samples, study results are occasionally contradictory. There have been several differences, particularly in terms of sampling methodology, storage, and molecular analysis, 2). Using salivary testing protocols for biomarker studies or other molecular analyses need to be optimised, standardized, and validated for better outcome and 3). The self-sampling or home-based saliva collection should be used and validated as soon as possible in drawing reliable conclusions for better clinical outcome.

Due to non-invasive, painless, sensitive and easy-to-collect nature of saliva, it can be utilised as an alternate approach for cost-effective screening, early disease diagnosis, effective monitoring, and posttreatment surveillance of HNSCC patients. It is a store house of various biomolecules such as nucleic acids (RNA, DNA, miRNAs), enzymes, proteins, exosomes, microorganisms and is a perfect non-invasive, easy and safe source of biological specimens for disease diagnosis and molecular biomarker discovery. Therefore, saliva-based liquid biopsy testing is the most preferable technique and opened-up a new avenue for screening of HNSCC patients. However, additional advanced studies are needed with large number of samples for optimization of saliva sample collection, preservation, and standardization of isolation methods for salivary biomarkers detection and widespread clinical applications.

Further, identifying the gaps in integrating parameters for broader applications in saliva-based diagnostics signifies a pivotal moment in the advancement of healthcare. It necessitates a concerted, multidisciplinary effort, underscored by long-term investment, standardization, and robust clinical validation. An imperative need for inter-institutional and inter-country collaborations as well as collaboration between clinical, academic research teams are most important. It is essential to harness the collective expertise, resources, and technological advancements available across borders and institutions to develop a saliva-based diagnostic test with unparalleled performance. Such collaborative efforts will not only facilitate the rapid advancement of science and technology but also ensure that the resulting test meets the stringent requirements for accuracy, efficacy, sensitivity, and specificity. By putting together, the brightest scientific minds and cutting-edge technologies, it is possible to expedite the development of a saliva test that not only surpasses the current standards but also becomes the most reliable and accessible tool for healthcare practitioners worldwide.

Author contributions statement

S.G. and P.K. participated in collection of relevant data and drafting the manuscript. B.C.D. conceptualized, designed and supervised, participated in data interpretation and critically reviewed, finalized and communicated the manuscript. All authors have read and approved the final manuscript.

Ethical approval

Not applicable.

Declaration of Competing Interest

The authors declare there is no conflict of interests.

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