Review

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Review

The Role of ctDNA and Liquid Biopsy in the Diagnosis and Monitoring of Head and Neck Cancer: Towards Precision Medicine

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Simple Summary: Liquid biopsy's use in the field of head and neck cancer has garnered interest due to providing an efficient and insightful alternative or complement to the present standards for the diagnosis and monitoring of patients. Certain biomarkers have been associated with specific cancer diagnoses, responses to treatment, and risk of recurrence, including circulating tumor DNA. Analysis of circulating tumor DNA and viral DNA via liquid biopsy has linked certain mutations and methylation patterns to various patient outcomes across several types of head and neck cancer. The present paper will review the recent literature examining liquid biopsy's clinical utility in head and neck oncology and will discuss the clinical implications of aspects of circulating tumor DNA, among other biomarkers.

Abstract: Recent data has shown a continued rise in the worldwide annual incidence and mortality rates of head and neck cancers. The present standard for diagnosis and monitoring for disease recurrence or progression involves clinical examination, imaging, and invasive biopsy techniques of lesions suspected of being malignant. In addition to limitations relating to cost, time, and patient discomfort, these methodologies have inherent inaccuracies for detecting recurrence. In view of these limitations, the analysis of patient bodily fluid samples via liquid biopsy proposes a cost-effective and convenient alternative, which provides insight on the biogenetic and biomolecular underpinnings of oncologic disease processes. Liquid biopsy's monitoring for biomarkers of head and neck cancer, including circulating tumor DNA, circulating tumor cells, and circulating cell-free RNA, has shown clinical utility in the screening, diagnosis, prognostication, and monitoring of patients with various forms of head and neck cancer. The present review will provide an update on the current literature examining the use of liquid biopsy in head and neck cancer care and the clinical applicability of potential biomarkers, with a focus on viral and non-viral circulating tumor DNA. Possible future avenues for research to address specific shortcomings of liquid biopsy will be discussed.

Keywords: head and neck cancer; liquid biopsy; ctDNA; squamous cell carcinoma; oropharyngeal carcinoma; nasopharyngeal carcinoma; genetic mutations; genomic sequencing; cancer biomarkers

1. Introduction

Head and neck cancer (HNC) refers to a pathologically varied group of malignant neoplasms of the upper aerodigestive tract and other structures of the head and neck regions [1]. Anatomic sites affected by HNC include the oral cavity, salivary glands, nasopharynx, oropharynx, nasal cavity, paranasal sinuses, larynx, skin of the head and neck and, depending on the clinical definition, the thyroid gland [2,3]. The global incidence of HNC is predicted to continue to rise over the coming years, with trends indicating that HPV-related oropharyngeal carcinomas will comprise the majority of HNCs over the next two decades [3]. From 2020 to 2022, cancers of the lip/oral cavity, larynx,

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nasopharynx, oropharynx, hypopharynx, and salivary glands rose from the 7th to the 6th most commonly diagnosed cancers and remained the 6th most common cancer to cause mortality worldwide. There are an additional 14,525 new cases and 14,876 deaths per year being recorded in 2022, compared to malignancies of other anatomical sites [4,5].

The presence of nucleic acids in blood plasma was first reported by Mandel and Metais in 1948 [6]. Cell free DNA (cfDNA) refers to genetic material organized in the form of DNA fragments of various sizes and from various origins found in bodily fluids independent of cells [7]. cfDNA can circulate free and unbound in the plasma, contained within a vesicle, or as part of macromolecular complexes composed of DNA bound to proteins or lipids [7]. DNA is released from cells into the plasma via several mechanisms, including apoptosis, necrosis, and NETosis, among others (Table 1) [8,9]. Given the role of cell damage and death in increasing the concentration of cfDNA in the serum, cfDNA has been recognized as a marker of inflammation and disease [9]. Accordingly, cfDNA has been found to be elevated in several pathological states, including certain kidney diseases, cardiovascular diseases, autoimmune conditions, and various cancers **[10–13]**.

Table 1. Current Standards for the Diagnosis of Head and Neck Cancers.

Type of Malignancy	Diagnostic Standard
Head and neck squamous cell carcinomas	Imaging, endoscopy, primary tumor biopsy and histological analysis, fine needle aspiration with ultrasound guidance in hard-to-access areas [14]
Nasopharyngeal carcinoma	Primary tumor biopsy with assistance via nasal endoscopy, imaging [15]
Salivary gland carcinomas	Fine needle aspiration and cytological analysis; if unclear, immunohistology of excised tumor tissue can establish diagnosis [16]
Sinonasal carcinomas	Imaging with both CT and MRI, endoscopy, primary tumor biopsy, "metabolic biopsy" via 18 F-FDG PET/CT [17]
Thyroid carcinomas	Fine needle aspirations with ultrasound guidance [18]

Circulating tumor DNA (ctDNA) was first explored by Leon et al. in 1977, and they found that participants with a variety of cancers had higher mean levels of cfDNA compared to healthy controls [13]. ctDNA refers to cfDNA derived from tumor cells [19]. ctDNA sequences are generally more fractionated and of shorter length than cfDNA fragments and are released from tumor cells into the bodily fluids either by cell death via apoptosis or necrosis or via active secretion [19]. Quantitative and qualitative changes in the characteristics of ctDNA can provide essential information on a patient's disease, thus having important clinical value (Table 2) [20]. For example, specific genetic mutations have been identified in various forms of cancer and can help inform diagnosis [20]. Methylation patterns have exhibited value in early detection of cancer, predicting response to treatment, and determining cell of origin and therefore, cancer stage [21,22]. The concentration of ctDNA can be indicative of tumor burden, tumor localization, and rate of tumor growth [23].

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Table 2. Identified Altered Genes in ctDNA by Cancer Type.

Type of Malignancy	Identified Altered ctDNA Genes
Head and neck squamous cell carcinomas	TP53, NOTCH1, CDKN2A, CALML5, DNAJC5G, LY6D, EDNRB, TIMP3, PCQAP/MED15, CDKN2B, DAPK1, MGMT, GSTP1, PRDM2, RASSF1, DLEC1, UCHL1, RARβ2, WIF1, DCC, MLH1, CDH1 [24–30]
Nasopharyngeal carcinoma	RASSF1, CDKN2A, CDKN2B, DLEC1, DAPK1, UCHL1, WIF1, RARβ2, CDH1, PLCB3, C18orf1, ZNF516, FGR, PLCB3, FGR, PRKCZ, KDM4B, HLX, ZNF516, MGRN1, UHRF1, SPI1, PLEC1, MPO, ADRBK1, COL11A2, MLLT1, FUT4, MBP, FLNB, SMTN, KCNT1, APEH, HLA-DRB5 [31–35]
Salivary gland carcinomas	TP53, PIK3CA, ERBB2, ATM, EGFR, HRAS, BRAF, KRAS, EGFR, CDK6 [36,37]
Sinonasal carcinomas	KRAS, NRAS [38,39]
Thyroid carcinomas	TP53, BRAF, RAS, RET, ALK, NTRK, PIK3CA, PTEN, RASSF1, SLC5A8 [18,40–46]

Liquid biopsy allows for the analysis of ctDNA and other biomarkers of cancer present in bodily fluids, including the blood, urine, saliva, and pleural, peritoneal, and cerebrospinal fluids [47]. The concept of liquid biopsy as an oncological diagnostic modality was first introduced in 2010 as a method for detecting circulating tumor cells (CTCs) and its application was expanded to the examination of ctDNA and other biomarkers in 2011 **[48–50]**. Regarding head and neck cancer, the current standard methods for diagnosis include endoscopy, imaging, and biopsy of a tumor sample by techniques such as excision or fine needle aspiration, while monitoring for recurrence is generally restricted to imaging and clinical examination [14,51,52]. The use of liquid biopsy has been successful in the diagnosis and monitoring of other solid tumors, and past studies on the implementation of liquid biopsy for the detection and monitoring of head and neck cancers have shown potential for its clinical adoption as a less-invasive and less-expensive alternative to the current diagnostic standards [51,52]. The present paper aims to review the literature on the implementation of liquid biopsy in head and neck cancer care, with a specific focus on ctDNA, and discuss its current and future use in relation to various histological types of head and neck malignancies.

2. Liquid Biopsy

Liquid biopsies started with isolation of CTCs but have since expanded to include ctDNA and circulating cell-free RNA (cfRNA) [53]. In oncology care, liquid biopsies are a minimally invasive method that can guide therapy, assess prognosis and tumor burden, and detect cancer by identifying and analyzing these molecules from a patient's bodily fluid, most often blood [54]. While blood is the most common sample for liquid biopsy, saliva, cerebrospinal fluid, ascites fluid, pleural fluid, and urine can be sampled as well.

The diagnostic workup for a patient with suspected head and neck cancer focuses on history, physical exam, and imaging with tissue biopsies or cytology to definitively diagnose a patient [55,56]. With the rapidly expanding field of genetic sequencing and tumor molecular profiling, liquid biopsies are being explored for their potential to improve outcomes for head and neck cancer patients *[55–57]*. In comparison to tissue biopsy or cytology, which are still the gold standards for diagnosing head and neck squamous cell carcinomas, liquid biopsies are minimally invasive and may allow for

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earlier detection, prognosis, and monitoring treatment response *[54–56]*. For head and neck cancers, blood, serum, or plasma is still the most common sample used but improving technology may support the use of saliva based liquid biopsies [55,56].

There are a wide variety of biomarker assays being studied including CTCs, ctDNA, cfDNA, exosomes (EXOs), and tumor metabolites. Due to their rarity, CTCs can be difficult to isolate, but new 2-step isolation and purification techniques have allowed for higher purity isolation of CTCs [56]. Other assays focus on non-cellular cancer components like ctDNA or cfDNA, which are released by cancer cells or by macrophages that phagocytose them [56]. Tests must account for the varying levels of cfDNA depending on tumor burden, stage, and treatment [56]. EXOs are extracellular vesicles released by cells that are within 30-150nm and can be found in most bodily fluids [56]. The proteins, miRNA, mRNA, and DNA contained within an EXO's lipid bilayer structure create a tumor's microenvironment and immune response [56]. EXO assays may have a higher yield than the other assays; however, they can be more time-consuming and contaminated [56]. Finally, tumor metabolites like stearyl alcohol, sucrose, and plasma lysophosphatidylcholines are being explored for their potential as cancer markers [56].

CTCs, while imperfect, have been studied for their ability to prognosticate disease-free survival and overall survival in head and neck cancer patients and may have improved accuracy when used together with ctDNA [56,58]. In addition to its use as a diagnostic and staging biomarker, ctDNA also has the potential to monitor patients after treatment to detect recurrence, possibly before a patient is symptomatic [56]. Rutkowski et al. showed that surveillance using a combination of ctDNA levels with PET-CT may improve detection of recurrence of HPV-positive oropharyngeal cancer [59]. Furthermore, Lele et al. found that ctDNA detection in patient blood samples was significantly associated with post-treatment head and neck squamous cell carcinoma (HNSCC) recurrence whereas identification of lesions with increased FDG uptake on PET scan was not, suggesting that liquid biopsy may be a superior modality for surveillance in certain cases [60].

In comparison to traditional tissue biopsy, which remains the gold standard for diagnosis, liquid biopsy is less invasive and can allow for earlier detection, monitoring throughout treatment, and earlier detection of recurrence. In early stages when malignancies may not have yet formed a discrete recurrence, tissue biopsy is not feasible. Moreover, pulmonary nodules up to one centimeter in size are often too small for biopsy. While liquid biopsy can facilitate earlier detection in these stages, the concentration of biomarkers like ctDNA will be low and is susceptible to more false positives [61]. In combination with tissue biopsy, ctDNA and CTCs may provide a more complete understanding of a tumor's genetic sequence to guide treatment plans [54]. For HPV-associated oropharyngeal squamous cell carcinomas that present with cystic or necrotic nodes, Ferrandino et al. report that while FNA only has a 70-80% success rate in identifying malignancy, ctDNA had a pooled sensitivity of 81% and specificity of 98% [62].

For patients receiving immune checkpoint inhibitor treatment, imaging studies may show a pseudo-progression of the tumor due to the increased inflammation. Studies across cancer types have shown that ctDNA and cfDNA levels could be monitored to assess treatment response to immune checkpoint therapy in ways that can be challenging for imaging studies [54]. For patients with positive ctDNA levels, use of HPV ctDNA identified treatment failure earlier on than imaging (MRI, CT, and 18 F-FDG PET-CT) [63].

3. Head and Neck Squamous Cell Carcinoma

HNSCC make up the majority of head and neck malignancies, with over 90% of HNCs being HNSCCs [24,64]. HNSCC can arise from the mucosal epithelium of the oral cavity, oropharynx, hypopharynx, larynx, and nasopharynx, with the main recognized risk factors for its development including tobacco use, alcohol use, and infection by high-risk human papillomavirus (HPV) or Epstein-Barr virus (EBV) [24,64,65]. HPV infection has been identified as driving the increase in rates of oropharyngeal cancers in developed countries, with HPV-16 being the most common HPV type identified on biopsies of HPV-positive HNCs [3,66].

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Early studies on the application of liquid biopsy to the treatment and monitoring of HNSCC found that CTCs were detectable in only a small number of cases, with increased stage and high tumor burden being associated with an increased number of CTCs in peripheral blood samples [67]. CTC counts were found to inform HNSCC tumor localization, potential dissemination secondary to treatment, and prognosis in terms of likelihood of disease-free survival, recurrence, and disease progression [67]. Studies on ctDNA found that increased ctDNA levels in samples also correlated with advanced stage and post-treatment recurrence, TP53 was the most commonly identified mutated gene in HPV-negative HNSCC, ctDNA was detected in saliva samples of all patients with oral HNSCC, and that combined plasma and saliva liquid biopsy was the most sensitive method for detecting ctDNA [67,68]. However, the paucity of data of early studies on liquid biopsy use in HNSCC diagnosis and monitoring due to small sample sizes and large variation in methodology implemented and patient populations examined limited the conclusions that could be drawn [67,68].

More recently, a systematic review by Huang et al. examining studies published between 2012 and 2023 corroborated earlier findings that TP53 was the most commonly mutated gene in HNSCC [24]. TP53 was also found to have the highest rate of concordant variants between tumor DNA (tDNA) and ctDNA at 6.25%, exhibiting the low degree of concordance in mutated genes detected in ctDNA and DNA of samples taken directly from the primary tumor site [24]. The highest degrees of concordance were detected in HPV-negative and stage IV HNSCCs [24]. The low concordance rate may be due to the high degree of intra-tumoral genomic heterogeneity between and within core and marginal sites of HNSCC tumors, with Payne et al. reporting that 96.5% of mutated genomic variants were found exclusively in a single site [69]. The examination of ctDNA via liquid biopsy in this same sample of patients was able to detect an average of over 79% of high-frequency genomic variations and most tumor site-specific mutations that could be missed by single-site tumoral biopsy, showcasing the potential of ctDNA liquid biopsy in both diagnosis and tailoring of treatment of HNSCC according to genomic patterns identified [69]. Additionally, serial liquid biopsy testing of patients showed how frequencies of genomic variants and levels of intra-tumoral heterogeneity changed leading up to and at HNSCC recurrence [69].

HPV-positive and HPV-negative HNSCCs differ in terms of commonly identified oncogenic mutations which serve as identifiable targets for liquid biopsy. Data from The Cancer Genome Atlas (TCGA) has indicated that PIK3CA was the most commonly mutated oncogene in HPV-positive HNSCC, whereas genomic alterations in HPV-negative HNSCC were mostly limited to tumor suppressor genes, including TP53, NOTCH1, and FAT1 mutations and CDKN2A inactivation *[70–74]*. While a previous study found that high PIK3CA copy number gain in HNSCC tumor samples were significantly associated with lower disease specific-survival and larger tumor volume, no studies have been found to date examining the prognostic value of PIK3CA mutations in the ctDNA of HNSCC patients [75]. The detection of TP53 mutations in ctDNA has been associated with decreased progression-free and overall survival, presence of disease at last visit, and regional metastasis, potentially making TP53 a valuable biomarker for prognostication [25,26]. While FAT1 mutations have been associated with better overall survival in HPV-negative HNSCC patients, its relationship to prognosis has not been examined in the exclusive context of ctDNA [76]. Lastly, CDKN2A and NOTCH1 ctDNA mutations were not found to have any prognostic value [25]. Further research exploring the role of specific mutations present in the ctDNA of HNSCC and other HNC patients and their relation to prognosis is warranted.

While PIK3CA mutations are more frequent in HPV-positive HNSCCs compared to HPV-negative HNSCC, it is also commonly mutated in HPV-negative HNSCC [77]. Another target for the detection of HPV-positive HNSCC via liquid biopsy is circulating tumor HPV DNA (ctHPVDNA), which results from the integration of HPV DNA into the genome of the host cell [78,79]. The use of digital droplet PCR to detect HPV-associated E7 exhibits greater efficiency and accuracy in diagnosis compared to tissue biopsy while also increasing the ability to discriminate HNSCC according to HPV status [78]. Ferrandino et al. found that the use of liquid biopsy to test for tumor tissue-modified viral-HPV DNA in peripheral blood samples to diagnose and test for recurrence of high-risk HPV-positive oropharyngeal SCC had sensitivity of over 88% and specificity of 100% [80]. Saliva samples from

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HPV-positive HNSCC patients have also shown increased ctHPVDNA levels and had high concordance rates with peripheral blood samples [81]. In addition to saliva and blood samples, urine is another bodily fluid in which ctDNA can be detected. While the use of urine samples has previously been limited to detection of cancers of the urinary tract, transrenal ctDNA, which refers to ctDNA that passes from the bloodstream to the urine via filtration by the kidneys, has been shown to have diagnostic value in HNSCC [82]. A recent study by Bhambhani et al. found that ultrashort fragments comprised of less than 50 base pairs, which are likely to be overlooked by traditional ctDNA assays, of HPV-16 transrenal ctDNA were detectable in patient urine samples by a newly developed ctDNA assay [82]. Testing for ctHPVDNA via liquid biopsy has been shown to have potential prognostic value, with ctHPVDNA levels exhibiting correlations with tumor burden, disease progression and metastasis, treatment response, residual disease, recurrence, and survival [79,81]. Preliminary data has also shown that ctHPVDNA has potential in the diagnosis of HPV-positive sinonasal and nasopharyngeal squamous cell carcinomas (SCC), which differ genotypically from HPV-positive oropharyngeal SCCs in that HPV-16 is less predominant compared to other HPV types [83].

E6 and E7, the two major HPV-related oncoproteins, affect DNA methylation patterns, with hypermethylation of specific genetic sequences being reported in HPV-positive HNSCC [84]. Of note, methylation of genes CALML5, DNAJC5G, and LY6D in ctDNA was highly effective in differentiating between HPV-positive oropharyngeal SCC patients and healthy controls, and is an identifiable target in ctDNA studies [27]. Specifically, hypermethylation of EDNRB in ctDNA was found to be significantly associated with HNSCC when comparing a pooled group of HPV-positive and HPV-negative HNSCC patients to healthy controls; however, hypermethylation of EDNRB was only detectable in a minority of HNSCC patients, limiting its value as a diagnostic biomarker [28]. When considering methylation patterns in ctDNA of salivary samples, Lim et al. found that genes RASSF1α, CDKN2A, TIMP3, and PCQAP/MED15 had higher levels of methylation in HPV-negative HNSCC patients compared to healthy controls, while the same genes had lower levels of methylation in HPV-positive HNSCC patients compared to healthy controls [29]. Other findings on methylation patterns in HNSCC previously described in the literature include associations between levels of primary tumor and ctDNA gene methylation levels, hypomethylation of Alu elements in ctDNA samples of HNSCC patients, and hypermethylation in ctDNA of HNSCC patients of gene promoter sequences of genes including CDKN2A, CDKN2B, DAPK1, MGMT, GSTP1, PRDM2, RASSF1, DLEC1, UCHL1, RARβ2, WIF1, DCC, MLH1, and CDH1 [30]. Further elucidation of ctDNA methylation patterns and their implications in the characterization of HNSCCs will further inform the development of future diagnosis and treatment modalities.

4. EBV+ Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is a rare cancer that originates from the mucosal epithelium of the nasopharynx and most commonly arises from the fossa of Rosenmüller [85]. Rates vary by geographical region, with over 70 to 80% of new cases being diagnosed in East and Southeast Asia [85,86]. Geographical differences in incidence and prevalence of NPC may be due to various environmental, genetic, viral, and dietary factors, as well as the interplay between them [87,88]. Of note, in endemic regions, EBV infection is implicated as contributing to carcinogenesis in about 96% of NPC cases [88].

Traditionally, NPC has been diagnosed via nasal endoscopy and direct sampling and biopsy of the lesion, followed by imaging scans for staging [15]. An early study examining cell-free EBV DNA (cfEBVDNA) was published in 1999, which found that cfEBVDNA was detectable via real-time quantitative PCR in the peripheral blood plasma samples of 55 of 57 patients with histologically confirmed diagnoses of NPC compared to 3 of 43 healthy controls [89]. Detected cfEBVDNA levels were significantly higher in patients with stage III/IV NPC compared to those with stage I/II NPC, and 7 of 15 patients demonstrated decreased cfEBVDNA following the completion of radiotherapy [89].

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The diagnostic and prognostic utility of plasma cfEBVDNA has been further highlighted by findings showing significant correlations between plasma cfEBVDNA concentrations and gross tumor volume of the primary lesion and metastatic lymph nodes [90]. Overall survival, disease-free survival, distant metastasis-free survival, and distant metastasis are all significantly associated with the detection of pretreatment plasma cfEBVDNA, while posttreatment plasma cfEBVDNA levels are significantly associated with distant metastasis and locoregional recurrence [90,91]. It should be noted that plasma cfEBVDNA was preferable to MRI in detecting distant metastasis, whereas MRI was preferable to cfEBVDNA in detecting locoregional occurrence, highlighting the complementary roles that liquid biopsy and MRI could play in post-treatment monitoring of NPC [91].

It has been demonstrated that testing plasma samples for cfEBVDNA carries potential utility in screening for early-stage NPC in Hong Kong, where NPC is endemic [92]. Chan et al. found that 34 of the 309 participants with persistently elevated levels of plasma cfEBVDNA were ultimately diagnosed with NPC. Screened patients were diagnosed with early-stage disease at a greater proportion than in a previous study, screening was associated with greater progression-free survival, and screening for NPC via plasma cfEBVDNA had a sensitivity and specificity greater than 97% [92]. To differentiate between individuals with and without NPC who tested positive for cfEBVDNA in the plasma, a follow-up study molecularly profiled cfEBVDNA and found that NPC patients had generally longer fragment lengths than their non-NPC counterparts [93]. While promising, it has been argued that screening for NPC via quantification of cfEBVDNA should be used in conjunction with other screening modalities: an estimated 130 patients with NPC would be missed annually in Hong Kong if plasma cfEBVDNA was implemented as the sole population screening tool [94].

Several DNA sequences have been identified as targets for hypermethylation in samples from patients with NPC. Higher rates of gene-silencing EBV-associated hypermethylation of CpG islands have been noted in the cfDNA promoter sequences of tumor suppressor genes RASSF1, CDKN2A, CDKN2B, DLEC1, DAPK1, UCHL1, WIF1, RARβ2, and CDH1 of NPC patients compared to healthy controls *[31–34]*. ctDNA analysis of peripheral blood samples shows the highest levels of methylation in patients with metastatic NPC of the gene bodies and intragenic regions of chromosomes 1 and 2, with greater hypermethylation levels of the open sea region compared to other regions of the CpG islands, which differs from DNA hypermethylation patterns of NPC tumor tissue [35]. More specifically, genes PLCB3, C18orf1, ZNF516, FGR, PLCB3, FGR, PRKCZ, KDM4B, HLX, ZNF516, MGRN1, UHRF1, SPI1, PLEC1, MPO, ADRBK1, COL11A2, MLLT1, FUT4, MBP, and FLNB where hypermethylated, whereas genes SMTN, KCNT1, APEH, and HLA-DRB5 were hypomethylated [35]. Quantitative PCR of NPC patient saliva samples was also able to reliably differentiate between patients with NPC and healthy controls on the basis of cfEBVDNA CpG island methylation levels, with decreases and increases in methylation levels relative to the cut off value of the assay after therapy and after recurrence, respectively [95]. Given these findings, hypermethylation patterns of ctDNA carry potential for prognosis in addition to diagnosis.

Other biomarkers for NPC include CTCs, whose levels in the peripheral blood have been found to positively correlate with N stage and overall clinical stage and negatively correlate with survival in patients with stage III-IVA NPC [96]. Post-chemoradiotherapy, mesenchymal CTC detection in peripheral blood samples was positively correlated with N stage and negatively correlated with 3-year survival [96]. MicroRNAs, which are approximately 22 nucleotide RNA fragments, encoding the BART region of EBV DNA are tissue-specific and can be released into the bloodstream similarly to ctDNA, with suggestions that BART microRNA could be used for the detection of NPC due to higher levels in the peripheral blood of NPC patients compared to healthy controls [15,97]. Lastly, there is recent evidence that tumor-educated platelet long non-coding RNA regulator of reprogramming plasma levels are negatively associated with NPC and carry a similar diagnostic value to cfEBVDNA [98].

5. Other Types of Head and Neck Cancers

Salivary gland primary tumors are rare and most commonly affect the parotid gland, although they can also arise from the smaller salivary glands in which case they are generally malignant [16].

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Diagnosis is done via fine needle aspiration and subsequent histological analysis, yet diagnosis can be challenging due to the histological heterogeneity of salivary gland neoplasms and the difficulty to differentiate between benign and malignant tumor cells on cytology [16]. Consequentially, determination of the neoplasm's histology is typically done post-surgery via immunohistology [16].

Several potentially targetable biomarkers for the detection and monitoring of salivary gland cancers via liquid biopsy have been explored; however, there is a paucity of previous literature on the topic. Genomic profiling of ctDNA in a set of patients with various histological types of salivary gland carcinomas determined the most commonly altered genes to be TP53, PIK3CA, ERBB2, ATM, EGFR and HRAS, while BRAF and KRAS mutations and EGFR amplification were identified as potentially targetable alterations that were newly detected on serial testing [36]. When examining ctDNA alterations by histological subtype of salivary gland carcinomas, it was found that PI3KCA mutations were common in adenoid cystic carcinoma and salivary duct carcinoma, ERBB2 mutations were common in salivary gland adenocarcinoma, and EGFR mutations were common in salivary gland mucoepidermoid carcinoma [36]. Additionally, in patients with metastatic adenoid cystic carcinoma, copy number analysis detected chromosomal alterations in ctDNA that were consistent with genetic mutations from samples taken from the primary tumor site, and CDK6 gene amplification in chromosome 7q was identified in peripheral blood samples as a potential biomarker for disease progression [37]. Other identified potential biomarkers include elevated plasma levels of IL-33 in benign and malignant salivary gland neoplasms and its receptor, sST2, among metastatic acini cell carcinoma and benign pleomorphic adenoma, elevated levels of IL-4 in the peripheral blood of patients with malignant salivary duct carcinomas, and elevated levels of CA 19-9 in saliva samples of patients with malignant parotid cancers [16]. Preliminary studies have also noted that increased levels of expression of androgen receptor splicing variant ARv7 by CTCs in peripheral blood samples of a single patient with metastatic salivary gland carcinoma predicted treatment resistance to combined androgen blockade therapy, and that detection of CTCs in peripheral blood samples of patients with adenoid cystic carcinoma could indicate local recurrence or distant metastasis [99,100].

Sinonasal cancers are rare tumors of the head and neck that may present with nasal and neurological symptoms and carry a significant risk of metastasis and local recurrence [17]. Current recommendations state that sinonasal cancers should be diagnosed via endoscopy and tumor biopsy followed by imaging with CT, MRI, and 18 F-FDG PET/CT to evaluate localization, histology, and size of the tumor, invasion into nearby bony and soft tissue structures, and presence of metastasis [17]. As noted in past literature, data on the utility of liquid biopsy in the diagnosis and monitoring of sinonasal cancers is sparse [101]. Micro RNA has been identified as a potential indicator of disease progression in patients with sinonasal intestinal-type adenocarcinomas (ITAC), with levels of miR-34c levels in nasal washings being increased in patients with increased levels of tumor differentiation and decreased in patients with signs of tumor intracranial extension, orbital extension, and advanced tumor staging [102]. Additionally, Cabezas-Camarero et al. discussed a case of the use of liquid biopsy to detect KRAS mutations in exon 2 codon 12 of CTC DNA in the peripheral blood of a patient with recurrent and anti-EGFR therapy-resistant ITAC, which were concordant with mutations found in the solid tumor biopsies via BEAMing, a technique that was previously found effective in detecting colorectal cancer-related genetic mutations [38]. A case series by Freiberger et al. on three patients with immunotherapy-resistant sinonasal melanoma found that NRAS mutations arose in the ctDNA collected from plasma samples during or after finishing treatment [39]. Lastly, two of three patients with sinonasal cancer who had CTCs detected in their peripheral blood samples had locally advanced sinonasal differentiated carcinoma [103]. While these findings may suggest promise in the use of liquid biopsy in the care of patients with sinonasal cancers, larger scale studies need to be carried out before broader conclusions are made.

Thyroid cancer refers to various malignancies that originate from thyroid tissue follicular cells or C-cells and differ regarding histology and clinical implications [40]. The current standard for the diagnosis of thyroid cancers is detection by ultrasound imaging followed by confirmation via fine-needle aspiration cytology (FNAC); however, in addition to being invasive, FNAC is operator-dependent, results in indeterminate findings in 15-30% of cases, and is limited in differentiating

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between benign follicular adenomas and malignant follicular carcinomas [18]. Monitoring for recurrence of thyroid cancer post-total thyroidectomy is done by serial measurements of serum thyroglobulin levels, but this is limited by variations in results of different assays and the interference of serum thyroglobulin antibody [18]. Liquid biopsy has the potential to address these limitations.

Genomic patterns of plasma ctDNA samples have been found to vary between patients with different types of thyroid cancer, with TP53 being the most commonly mutated across histological subtypes [40]. Other altered genes included BRAF, RAS, RET, ALK, NTRK, PIK3CA, and PTEN [18,40,41]. Specifically, one study reported that the BRAFV600E mutation has been noted in the serum samples of some patients with papillary thyroid cancer but was not found to be associated with lymphatic invasion, lymph node metastasis, or extra-nodal extension [42]. Conversely, the prognostic utility of BRAFV600E mutated ctDNA is supported by the findings of Almubarak et al., who found that ctDNA levels were higher in patients with metastatic compared to non-metastatic papillary thyroid cancer [43]. Additionally, ctDNA serum levels were found to have a higher sensitivity and specificity (86% and 90%, respectively) in diagnosing papillary thyroid cancer than the standard thyroglobulin assay (78% and 65%, respectively) [43]. Hypermethylation of RASSF1 and SLC5A8 promoter regions was found to be higher in both tumor tissue samples and ctDNA of patients with papillary thyroid cancer compared to those with benign thyroid nodules, and hypermethylation of the promoter regions of SLC5A8 in ctDNA was significantly associated with stage of papillary thyroid cancer [44]. Another study reported that concordance of BRAFV600E mutated plasma ctDNA and primary lesion DNA collected via FNAC was noted in 73.08% of a cohort of participants composed of 22 patients with papillary thyroid cancer and 4 comparators with benign thyroid nodules, and that 5 of 6 patients with papillary thyroid cancer that were positive for BRAFV600E mutated ctDNA were found to be negative 24 hours post-surgery [45]. Yet, this is not the case across thyroid cancer subtypes: A study on patients diagnosed with medullary thyroid cancer found that detection of mutually exclusive RET and RAS mutations in ctDNA did not significantly associate with patients being pre- or post-surgery [46]. In terms of anaplastic thyroid carcinoma, the most aggressive subtype of thyroid cancer, Sandulache et al. reported that the most commonly detected ctDNA mutations in a sample of 23 patients were TP53 and BRAF (65% and 48%, respectively), and that treatment-naive patients had higher concordance rates between ctDNA and tDNA [41].

Apart from ctDNA, other biomarkers that can be targeted by liquid biopsy include CTCs and various types of cfRNA. CTC detection and monitoring has been claimed to be effective in distinguishing between benign and malignant thyroid nodules, distinguishing between patients with differentiated thyroid cancer and healthy controls, determining initial tumor stage, prognostication in patients with metastatic disease, determination of primary tumor size and presence of vascular invasion, and monitoring patient response to radioiodine therapy [18,104]. cfRNA had utility in diagnosis, identification of recurrence, staging, prognostication, and informing treatment in several types of thyroid cancer [18].

6. Challenges and Limitations

Although promising, the use and implementation of liquid biopsy in head and neck oncology care is not without challenges and limitations. For example, high costs and convoluted data analyses of certain technologies, such as droplet digital PCR and next generation sequencing, present a barrier for the widespread clinical adoption of liquid biopsy [105]. Other issues relating to costs include the need for adequate materials and infrastructure for the storage of patient samples, which could affect the reliability and accuracy of liquid biopsy if suboptimal [106].

Another barrier that has been identified is the necessity for more data from prospective, longitudinal studies on the clinical utility of liquid biopsy to ascertain the effectiveness of various assays in patient care [107]. Such studies could help refine the sensitivity and specificity values of these assays, which have been noted by some to be clinically suboptimal [71,108]. Furthermore, in specific relation to diagnosis and monitoring of HPV-related HNSCC, improvement of current technologies is warranted to expand the currently limited set of HPV types that can be detected via

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liquid biopsy assays [109]. The lack of FDA-approved, head and neck cancer-related liquid biopsy biomarkers poses another challenge that prevents its adoption for clinical use [71].

Other considerations to note concern disease-, treatment-, and data collection-related limitations of liquid biopsy. Due to the tumor heterogeneity-related discordance often found between circulating biomarkers and those of the primary tumor tissue, liquid biopsy may be limited in its ability to comprehensively characterize malignancies from which the analyzed biomarkers originate [69,105]. Furthermore, recurrence at primary sites may be less detectable, especially in the setting of altered lymphatics. Additionally, variables including undergoing radiochemotherapy, gastrostomy tube placement, having an infection, and antibiotic use were associated with increased cfDNA levels in blood samples of head and neck cancer patients, which could be confounding variables in the use of liquid biopsy for the monitoring of treatment response and cancer recurrence [110]. Furthermore, the capability of liquid biopsies to distinguish between biomarkers originating from cancerous and non-cancerous tissues poses another hurdle [108].

Finally, the management algorithm for patients with early recurrences detected via liquid biopsy without clinical or radiographic evidence of disease has yet to be established. Haring et al. argue that liquid biopsy may be limited in its ability to characterize the burden or location of disease recurrence [111]. For example, there are no agreed upon ctHPVDNA levels to differentiate between local, regional, and distant recurrences [112]. This limits the effectiveness of liquid biopsy in informing therapeutic intervention, which differ in appropriateness depending on location of recurrence [111]. In terms of surveillance schedules, positive liquid biopsy testing without clinicoradiological correlates can prompt more frequent testing and imaging to ensure earlier detection of clinical disease [113]. Clinical trials and other future investigations will quantify the utility of liquid biopsy in this regard.

7. Conclusion and Future Directions

Liquid biopsy carries the potential to revolutionize head and neck cancer care. It has shown promise in diagnosis, staging, prognosis, and treatment monitoring of various types of head and neck cancers; however, more research is warranted to resolve contradictions presented by the results of different studies and elucidate liquid biopsy's clinical applicability and utility. Future avenues for investigative studies include determining ways to simplify and increase accessibility and affordability of liquid biopsy assays, refining current technologies to ensure adequate clinical standards are met, and identifying other disease-specific biomarkers and elucidating the implications of their detection at different points in a patient's disease course. By providing insight into the biomolecular basis of individual patients' disease processes, liquid biopsy promises to open possibilities for individualized and personalized approaches to oncologic patient care.

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