



# Liquid Biopsy in Head and Neck Cancer: Current Evidence and Clinical Applications



Julide Kasaboglu<sup>1</sup>, Spiridon Todorov<sup>1</sup>, Tsvetomir Marinov<sup>2</sup>

*1. Department of ENT, University Hospital "Queen Joanna-ISUL", Medical University-Sofia, Sofia*

*2. Department of Anesthesiology and Intensive Care, University Hospital "Queen Joanna-ISUL", Medical University-Sofia, Sofia*

## Abstract

**Background:** Head and neck squamous cell carcinoma (HNSCC) is a biologically heterogeneous malignancy with substantial morbidity and high rates of locoregional recurrence despite advances in surgery, radiotherapy, and systemic therapy. Conventional diagnostic and surveillance approaches rely on tissue biopsy and imaging, which are invasive, spatially limited, and often insufficiently sensitive for detecting minimal residual disease or early relapse. Liquid biopsy has emerged as a minimally invasive strategy that enables real-time assessment of tumor burden and molecular dynamics through analysis of tumor-derived material in body fluids.

**Objective:** This review summarizes current liquid biopsy strategies in head and neck cancer, highlighting their biological foundations, analytical methodologies, and clinical applications across the disease continuum.

**Methods:** A narrative review of the literature was conducted, focusing on circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) with biological basis of liquid biopsy. Evidence from translational studies, clinical cohorts, and emerging prospective trials was critically evaluated.

**Results:** Among liquid biopsy modalities, ctDNA represents the most clinically mature approach, demonstrating utility in treat-

ment response assessment, detection of minimal residual disease, and early identification of recurrence. Viral ctDNA, particularly human papillomavirus (HPV) DNA in oropharyngeal cancer, offers exceptional specificity and prognostic value. Circulating tumor cells provide complementary cellular and functional insights into tumor heterogeneity, metastatic potential, and treatment resistance, although technical challenges limit their routine use. Extracellular vesicles and non-coding RNAs show promise as diagnostic and prognostic biomarkers but require further standardization and validation. Saliva-based assays represent an attractive proximal sampling strategy, especially for HPV-associated disease.

**Conclusion:** Liquid biopsy strategies offer transformative potential in the management of head and neck cancer by enabling non-invasive, dynamic monitoring of disease biology. While ctDNA and viral biomarkers are closest to clinical integration, emerging multi-analyte approaches incorporating CTCs and extracellular vesicles may further enhance precision oncology. Prospective trials demonstrating clinical utility and outcome benefit are essential to establish liquid biopsy as a routine component of head and neck cancer care.

## 1. Introduction

Head and neck cancers account for over 900,000 new cases annually worldwide, with HNSCC constituting more than 90% of diagnoses [1]. Despite advances in multimodal therapy, including surgery, radiotherapy, chemotherapy, and immune checkpoint inhibitors, survival outcomes have improved only modestly over the past decades. Treatment-related morbidity is substantial, and disease recurrence – often aggressive and difficult to salvage – remains a major cause of mortality.

A critical limitation in contemporary management is the inability to accurately and noninvasively monitor tumor dynamics over time. Tissue biopsy, although essential for diagnosis, samples only a

small portion of a spatially and temporally heterogeneous tumor and cannot be repeated frequently. Imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) are indispensable but lack sensitivity for microscopic disease and are often confounded by post-therapeutic changes [2].

Liquid biopsy has emerged as a powerful concept that addresses many of these shortcomings. By analyzing tumor-derived components circulating in blood or present in proximal fluids such as saliva, liquid biopsy offers the potential for longitudinal disease monitoring, early detection of recurrence, assessment of treatment response, and characteri-

zation of tumor evolution under therapeutic pressure. Head and neck cancer represents a particularly compelling context for liquid biopsy due to the accessibility of proximal fluids and the presence of virally driven disease subsets.

## 2. Biological Basis of Liquid Biopsy

### 2.1. Conceptual framework of liquid biopsy

Liquid biopsy is founded on the biological principle that malignant tumors continuously release cellular and acellular components into body fluids throughout their development and progression. This phenomenon occurs from early tumorigenesis and intensifies with increasing tumor burden, invasiveness, vascularization, and metastatic dissemination. Unlike conventional tissue biopsy – which provides a spatially restricted and temporally static snapshot – liquid biopsy enables dynamic, systemic interrogation of tumor biology over time [1, 2].

In head and neck squamous cell carcinoma (HNSCC), this paradigm is particularly relevant. Tumors arise in anatomically complex regions where repeated tissue sampling may be impractical or associated with significant morbidity. Moreover, HNSCC exhibits pronounced spatial and temporal heterogeneity driven by clonal evolution, therapy-induced selective pressure, and interactions with the immune microenvironment. Liquid biopsy has the theoretical advantage of capturing this heterogeneity by integrating molecular signals derived from both primary and metastatic lesions [3].

### 2.2. Mechanisms of tumor-derived material release

Tumor-derived analytes detected by liquid biopsy originate through several complementary biological mechanisms:

#### Apoptosis and necrosis:

High proliferative activity and genomic instability lead to increased tumor cell turnover. Apoptotic and necrotic cell death result in the release of fragmented DNA and RNA into the extracellular space, which subsequently enter the circulation as cell-free nucleic acids. These fragments are typically short (~160–180 bp), reflecting nucleosomal packaging during apoptosis [4].

#### Active secretion via extracellular vesicles:

Viable tumor cells actively secrete extracellular vesicles (EVs), including exosomes (30–150 nm) and microvesicles. EVs function as mediators of intercellular communication and carry tumor-derived DNA, mRNA, microRNAs, long non-coding RNAs, proteins, lipids, and metabolites. Importantly, EV cargo is selectively packaged rather than passively released, providing biologically meaningful information about tumor signaling pathways and microenvironmental interactions [5, 6].

#### Cellular dissemination:

Invasive tumor cells may detach from the primary tumor mass and intravasate into blood or lymphatic vessels as circulating tumor cells (CTCs). This process is facilitated by epithelial–mesenchymal transition (EMT), extracellular matrix remodeling, angiogenesis, and immune evasion. CTCs represent viable cellular units with metastatic potential and provide insights into tumor aggressiveness and resistance mechanisms [7].

#### Iatrogenic release:

Diagnostic biopsy, surgical manipulation, radiotherapy, and chemotherapy can transiently increase tumor shedding into circulation. While this may enhance analyte detectability, it also introduces temporal variability that must be considered when interpreting liquid biopsy results [2].

### 2.3. Biology of circulating tumor DNA

Circulating tumor DNA (ctDNA) constitutes a variable fraction of total cell-free DNA (cfDNA) in plasma. In healthy individuals, cfDNA is predominantly derived from hematopoietic cells, whereas in cancer patients a subset originates from tumor cells. ctDNA contains tumor-specific somatic alterations, including point mutations, insertions/deletions, copy number alterations, chromosomal rearrangements, and epigenetic modifications [1, 4].

In HNSCC, ctDNA commonly harbors mutations in genes such as **TP53**, **PIK3CA**, **CDKN2A**, **NOTCH1**, and **FAT1**, reflecting the genomic landscape of squamous carcinogenesis. Importantly, ctDNA captures molecular heterogeneity across tumor sites, often revealing subclonal alterations not detected in primary tumor biopsies [8].



The proportion of ctDNA within cfDNA—referred to as tumor fraction—is influenced by tumor size, vascularity, proliferation rate, anatomical location, immune-mediated clearance, and treatment status. Early-stage disease and minimal residual disease (MRD) are characterized by extremely low tumor fractions (<0.1%), posing major analytical challenges and necessitating highly sensitive detection technologies [9].

#### 2.4. Fragmentation patterns and epigenetic features

Beyond sequence-level mutations, ctDNA exhibits distinctive **fragmentation patterns** and **epigenetic signatures** that reflect its tumor origin. Tumor-derived DNA fragments tend to be shorter and show altered nucleosomal footprints compared with cfDNA from normal tissues. These properties have been exploited in fragmentomics-based assays to improve cancer detection sensitivity [10].

Similarly, DNA methylation patterns are highly tissue- and cancer-specific. Methylation-based liquid biopsy assays can detect tumor-derived cfDNA even when mutation-based approaches fail, offering a complementary strategy for early detection and tissue-of-origin inference in HNSCC [11].

#### 2.5. Viral nucleic acids as liquid biopsy targets

A unique biological aspect of head and neck cancer is viral oncogenesis, particularly **human papillomavirus (HPV)**–associated oropharyngeal squamous cell carcinoma. In these tumors, viral DNA is clonally integrated into the host genome and released into circulation as part of ctDNA.

Circulating tumor HPV DNA (ctHPV DNA) has several biological advantages as a biomarker: it is tumor-specific, clonally stable, and not confounded by background somatic mutations or clonal hematopoiesis. These features confer exceptionally high analytical specificity and enable sensitive detection of disease dynamics [12].

Clinical studies demonstrate that ctHPV DNA levels decline rapidly with effective treatment and that persistent or rising levels strongly predict recurrence, often preceding radiographic detection by several months [13]. Detection of HPV DNA in saliva further exploits the anatomical proximity of oropharyngeal tumors to the oral cavity, although

local inflammation and oral microbiota may influence assay performance [14].

#### 2.6. Biology of circulating tumor cells

Circulating tumor cells represent intact malignant cells shed into the bloodstream and provide a cellular complement to ctDNA analysis. Unlike ctDNA, which reflects cumulative tumor burden, CTCs offer insights into cellular phenotype, protein expression, and functional behavior.

CTCs in HNSCC often exhibit heterogeneous phenotypes, including epithelial, mesenchymal, and hybrid EMT states. This heterogeneity reflects tumor plasticity and complicates detection using epithelial markers alone. Importantly, subsets of CTCs display stem cell–like properties, resistance to apoptosis, and enhanced metastatic potential, underscoring their biological relevance beyond simple enumeration [7, 15].

#### 2.7. Biological limitations and confounding factors

Despite its promise, liquid biopsy is subject to important biological limitations:

- Low analyte abundance in early-stage disease and MRD
- Biological noise from cfDNA released by normal tissues
- Clonal hematopoiesis of indeterminate potential (CHIP), which can introduce false-positive somatic mutations unrelated to cancer
- Heterogeneous shedding rates across tumor sites and disease states

These factors necessitate careful assay design, matched white blood cell sequencing, and cautious clinical interpretation [9, 16].

### 3. Circulating Tumor DNA (ctDNA)

#### 3.1. Characteristics and detection methods

Circulating tumor DNA is the tumor-derived fraction of cell-free DNA (cfDNA), consisting of short DNA fragments released into the bloodstream. ctDNA harbors tumor-specific genetic and epigenetic alterations, including somatic mutations, copy number changes, and methylation patterns [4].

**Detection strategies include:**

- **Tumor-informed assays**, which track patient-specific mutations identified from tumor tissue
- **Tumor-naïve assays**, which use predefined gene panels or methylation signatures
- **Droplet digital PCR (ddPCR)**, offering high sensitivity for known targets
- **Next-generation sequencing (NGS)**, enabling broader molecular profiling

Each approach involves trade-offs between sensitivity, breadth, cost, and turnaround time [5].

**3.2. Clinical evidence in HNSCC**

Multiple studies have demonstrated the feasibility of ctDNA detection in HNSCC, with detection rates correlating with tumor stage, burden, and anatomical site [6]. ctDNA levels decline following successful treatment and rise with disease progression, supporting its role as a dynamic biomarker.

**Minimal residual disease (MRD)**

MRD detection represents one of the most promising clinical applications of ctDNA. Post-treatment ctDNA positivity has been associated with significantly increased risk of recurrence following surgery or chemoradiation [7]. In several studies, ctDNA detected molecular relapse months before radiographic or clinical evidence, offering a potential window for earlier intervention.

However, the clinical value of earlier detection remains contingent on the availability of effective salvage strategies and on demonstrating that ctDNA-guided interventions improve outcomes.

**4. Viral ctDNA in HPV-Associated Head and Neck Cancer**

HPV-positive oropharyngeal squamous cell carcinoma represents a biologically distinct subset of HNSCC with favorable prognosis and unique viral biomarkers. Circulating tumor HPV DNA (ctHPV DNA) can be detected in plasma and saliva with high specificity [8].

Clinical studies demonstrate that clearance of ctHPV DNA during treatment correlates with response, while persistent or rising levels strongly predict recurrence [9]. Saliva-based assays are par-

ticularly attractive due to anatomical proximity and noninvasive sampling.

Despite strong prognostic value, optimal thresholds, sampling intervals, and clinical response algorithms remain areas of active investigation.

**5. Circulating Tumor Cells (CTCs)****5.1. Biological significance of CTCs in head and neck cancer**

Circulating tumour cells (CTCs) are intact malignant cells that detach from the primary tumour or metastatic deposits and enter the bloodstream. Unlike ctDNA, which reflects fragmented genetic material, CTCs represent viable cellular units capable of seeding distant metastases and contributing to disease progression. Their presence reflects an active biological process involving tumour invasion, epithelial–mesenchymal transition (EMT), intravasation, immune evasion, and survival under hemodynamic stress [10].

In head and neck squamous cell carcinoma (HNSCC), CTC dissemination is influenced by tumour vascularity, local inflammation, hypoxia, and interactions with the tumour microenvironment. EMT plays a particularly important role, enabling epithelial tumour cells to acquire mesenchymal features that facilitate motility and resistance to apoptosis. This phenotypic plasticity contributes to the marked heterogeneity of CTC populations detected in HNSCC and complicates their identification using epithelial markers alone.

**5.2. Methods for CTC detection and isolation**

CTC detection remains technically challenging due to their extreme rarity, often occurring at frequencies as low as 1–10 cells per  $10^9$  blood cells. Current isolation strategies fall into two broad categories:

**1. Marker-dependent (immunoaffinity-based) methods**

These approaches rely on expression of epithelial markers such as EpCAM and cytokeratins. The CellSearch® system is the most widely used platform and the only FDA-cleared CTC assay in solid tumors. However, its reliance on epithelial markers limits sensitivity in HNSCC, where EMT-associated marker loss is common.



## 2. Marker-independent (physical property-based) methods

These techniques isolate CTCs based on size, deformability, density, or electric charge (e.g., microfluidic devices, filtration-based systems). Marker-independent platforms may capture a broader spectrum of CTC phenotypes, including mesenchymal and hybrid epithelial–mesenchymal cells, but often at the cost of reduced specificity.

Recent advances combine immunophenotyping with microfluidic enrichment and single-cell sequencing, enabling deeper molecular characterization of CTCs while partially overcoming limitations of older platforms.

### 5.3. Clinical evidence of CTCs in HNSCC

Numerous studies have evaluated the prognostic significance of CTCs in HNSCC. Meta-analyses and cohort studies consistently demonstrate that CTC detection correlates with:

- Advanced tumor stage
- Presence of nodal or distant metastases
- Poor overall survival and disease-free survival

CTCs have been detected both at baseline and during treatment, with persistence after surgery or chemoradiation associated with increased risk of recurrence. Importantly, CTC presence may reflect aggressive tumor biology rather than tumor burden alone.

However, detection rates in localized HNSCC remain variable, ranging from 10% to 40% depending on tumor site, disease stage, and assay methodology. This variability limits the reliability of CTCs as a standalone biomarker for early disease detection.

### 5.4. Functional and molecular characterization of CTCs

One of the principal advantages of CTC analysis over ctDNA lies in the ability to perform **cellular and functional studies**. CTCs can be interrogated for:

- Protein expression (e.g., PD-L1, EGFR)
- Genomic alterations at the single-cell level
- Transcriptomic and epigenetic profiles
- Stemness and EMT markers

In HNSCC, several studies have reported PD-L1 expression on CTCs, raising interest in their potential role as a predictive biomarker for response to immune checkpoint inhibitors. Additionally, molecular discordance between CTCs and primary tumor tissue has been documented, suggesting that CTCs may better represent therapy-resistant clones.

Experimental work has also demonstrated that subsets of CTCs exhibit stem cell–like properties, increased metastatic potential, and resistance to chemotherapy and radiotherapy, reinforcing their biological relevance beyond simple enumeration.

### 5.5. CTC clusters and metastatic potential

Beyond single CTCs, circulating tumor cell clusters – aggregates of tumor cells sometimes accompanied by stromal or immune cells – have emerged as highly metastatic entities. Although less frequently studied in HNSCC than in breast or colorectal cancer, preliminary evidence suggests that CTC clusters may carry disproportionately high metastatic potential and enhanced resistance to shear stress and immune clearance.

The clinical relevance of CTC clusters in head and neck cancer remains incompletely defined, but their detection could provide additional prognostic information and insight into metastatic mechanisms.

### 5.6. Limitations of CTC-based liquid biopsy in HNSCC

Despite their biological appeal, several limitations restrict the clinical applicability of CTCs in head and neck cancer:

- Low detection rates in early-stage disease
- High technical complexity and cost
- Lack of standardized isolation and enumeration methods
- Phenotypic heterogeneity due to EMT
- Limited reproducibility across platforms and centers

Compared with ctDNA, CTC assays are less scalable, require specialized infrastructure, and have not yet demonstrated sufficient sensitivity or clinical utility for routine surveillance.

## 6. Extracellular Vesicles, microRNAs, and cfRNA

Extracellular vesicles (EVs), including exosomes, carry DNA, RNA, proteins, and metabolites reflective of tumor biology and tumor–microenvironment interactions [11]. EV-derived microRNAs have been proposed as diagnostic and prognostic biomarkers in oral cavity, laryngeal, and nasopharyngeal cancers.

While promising, EV-based assays face significant methodological challenges, including heterogeneity of vesicle populations, isolation variability, and limited reproducibility across cohorts. To date, these platforms lack the level of validation required for routine clinical use.

## 7. Proximal Liquid Biopsy: Saliva and Oral Rinses

Saliva is a uniquely valuable specimen in head and neck cancer due to direct contact with tumor-bearing mucosa. Salivary liquid biopsy has demonstrated utility for detecting HPV DNA, somatic mutations, and epigenetic alterations [12].

However, inflammatory conditions, oral microbiota, and variability in sampling methods introduce confounding factors. Standardization of collection, processing, and normalization protocols is essential for broader adoption.

## 8. Clinical Applications and Limitations

### 8.1. Early detection

While liquid biopsy holds theoretical promise for early cancer detection, low ctDNA abundance in early-stage disease and the requirement for extremely high specificity limit current feasibility for population screening [5].

### 8.2. Treatment response and resistance

Serial ctDNA monitoring can reflect treatment response and may help distinguish true progression from post-treatment changes or immunotherapy-related pseudoprogression [13]. ctDNA profiling also enables detection of emerging resistance mutations in recurrent or metastatic disease.

### 8.3. Analytical and biological challenges

Key limitations include:

- Low tumor fraction in early disease
- Pre-analytical variability
- False positives due to clonal hematopoiesis
- Lack of standardized thresholds
- Unclear clinical actionability in many scenarios

## 9. Future Directions

Future progress in liquid biopsy for head and neck cancer will depend on:

1. Prospective trials demonstrating outcome benefit from ctDNA-guided management
2. Development of multi-analyte approaches combining ctDNA, methylation, and EV markers
3. Improved integration with imaging and clinical risk models
4. Cost-effectiveness and implementation science

## 10. Conclusion

Liquid biopsy represents a transformative but still evolving tool in head and neck oncology. ctDNA—particularly tumor-informed assays and viral ctDNA in HPV-associated disease—offers the most immediate clinical promise for MRD detection and surveillance. However, widespread adoption requires rigorous validation, standardized methodologies, and clear demonstration of clinical utility. As these challenges are addressed, liquid biopsy is poised to become a central component of precision medicine in head and neck cancer.

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# March 14, 2025

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Make Sleep Health a Priority

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**The celebration will take place in the Affiliates of Medical University - Varna in Shumen, Veliko Tarnovo and Sliven.**

**In the period March 04 - March 21, 2025 free diagnostic and consultative examinations of people with sleep problems will be held on the territory of the University Medical and Dental Center, Faculty of Dental medicine, Medical University - Varna.**



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