

Review

## Using Liquid Biopsy to Predict Relapse After Radiotherapy in Squamous Cell Head-Neck and Esophageal Cancer

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**Abstract.** Circulating cell-free DNA (cfDNA) in the blood of cancer patients contains tumor-specific mutated genes and viral genome that can be identified and quantified as 'tumor-specific cfDNA' (circulating tumor DNA, ctDNA). Various technologies are available that offer reliable detection of ctDNA at a low concentration. Quantitative and qualitative analysis of ctDNA may be of prognostic and predictive value in oncology. Here, we present concisely the experience on the assessment of ctDNA levels and kinetics during therapy in the outcome of radiotherapy (RT) and chemo-radiotherapy (CRT) in squamous cell head-neck cancer and esophageal squamous cell cancer patients. The levels of circulating viral (human papilloma virus or Epstein-Barr) ctDNA, and levels of total, mutated or methylated ctDNA at diagnosis are linked with tumor burden and clinical aggressiveness, and may be of prognostic or even predictive value of RT/CRT efficacy. Persistent ctDNA levels after therapy seem to predict high rates of tumor relapse several months before radiological documentation. This can prove of value for the identification of subgroups of patients who could benefit from RT dose-escalation or consolidation chemotherapy and immunotherapy, a hypothesis that should be tested in clinical trials.

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Squamous cell head-neck cancer (SCHNC) is a common malignancy linked etiologically with smoking habits and human papilloma virus (HPV) infection (1). Epstein-Barr virus (EBV) infection is also related to nasopharyngeal carcinomas, more often, of non-keratinizing histology (2). Surgery with postoperative radiotherapy (RT) or radical chemo-RT (CRT) for inoperable locally advanced disease provides a high overall curability in 67% of cases (3). Locoregional relapse remains the main cause of death in patients with SCHNC, as distant metastases occur in less than 30% of patients (4, 5). In contrast to the overall high cure rates offered by RT for SCHNC patients, locally advanced esophageal squamous cell cancer (ESCC) has high mortality (6). Radical or preoperative CRT is considered the standard therapeutic approach, offering 2-year survival rates of 35-40% (7).

Monitoring the persistence of residual disease using computerized tomography or magnetic resonance imaging (CT/MRI) and positron emission tomography (PET-CT) after surgery or RT/CRT for SCHNC is important, as salvage therapy may improve control of the disease. Detectable post-irradiation tumor in PET-CT is a main determinant factor of relapse and prognosis (8). Micrometastatic or micro-residual disease may, however, escape the detection ability of radiological imaging. Blind biopsies from the primary site are difficult to perform, confer significant discomfort to patients, and can easily miss the site of residual tumor. Methods to detect remnant SCHNC based on liquid biopsies, thus plasma or saliva used for the detection of circulating tumor DNA or RNA have emerged as promising tools that are currently under clinical investigation (9-11).

Circulating cell-free DNA (cfDNA) in the blood of cancer patients corresponds to fragmented DNA released by normal and cancerous tissues (DNA size of 100-800 base pairs) (12). Host, mitochondrial and viral DNA is released from cells undergoing various death pathways, including apoptosis and necrosis, or as a result of extracellular excretion through vesicles (13). High rates of proliferation and death that characterize cancer tissues

are the cause of increased cfDNA content in the blood of patients. Such DNA fragments contain tumor-specific mutated genes and viral genome that can be identified and quantified as 'tumor-specific cfDNA' (circulating tumor DNA, ctDNA) (14). Whether genetic analysis from ctDNA provides comparable results to DNA analysis from circulating tumor cells has not been thoroughly investigated. In a comparative study by Lee *et al.*, analysis of 20 pairs of cfDNA and circulating tumor cell DNA showed partial overlapping, while a significant number of genetic variants were unique for each one of the examined types of samples (15). Authors suggested that a combinatory analysis could improve the sensitivity of liquid biopsies, but further investigation is demanded.

Various technologies are available that offer reliable detection of ctDNA at a low concentration (16). Real-time PCR, digital PCR, and next-generation sequencing (NGS) are among the widely applied. Digital PCR, including droplet digital PCR and BEAMing (beads, emulsions, amplification, and magnetics), is used to detect point mutations at very low allele fractions. NGS is founded on the detection and analysis of millions of short DNA sequences, with high sensitivity and specificity. Analysis of data from NGS demands bioinformatics to allow detection of single nucleotide polymorphisms (SNPs), insertions and deletions (indels) or copy number aberrations (CNAs).

The aim of the current short review is to present concisely the experience on the assessment of ctDNA levels and kinetics during therapy in the outcome of RT/CRT in SCHNC and ESCC patients. The literature search was performed in the EMBASE and MEDLINE databases using the text-words: "radiotherapy", "head neck cancer", "esophageal cancer", "circulating DNA", "cell-free DNA", "ctDNA". Clinical studies focusing on the cfDNA and ctDNA quantification and their kinetics and correlation with RT outcome were included.

### Non-specific cfDNA

Although blood circulating and saliva residing DNA fragments contain tumor-specific mutated genes and viral genome fragments that can be quantified as tumor-specific ctDNA, the total quantity of the non-specific cfDNA content can be used as a marker of tumor aggressiveness. High amounts of DNA fragments released in the body fluids and plasma may indicate high proliferation and death of cancer cells, necrosis, or other biological pathways linked to tumor aggressiveness, and, moreover, persistent cfDNA content may indicate lack of tumor regression during therapy. Indeed, several studies suggest that cfDNA level reduction predict better chemotherapy outcome in patients with gastric, pancreatic, and lung cancer (17-19).

Mazurek *et al.* reported a study on the cfDNA levels in 200 patients with SCHNC, showing that high levels characterized patients with advanced stage and lymph node

metastasis (20). A small study by Verma *et al.* on 24 SCHNC patients undergoing CRT displayed that increasing cfDNA levels after the end of therapy defined clinically residual disease (21). Moreover, a report on 61 patients with ESCC treated with CRT showed that although cfDNA levels were not related with response to therapy, low short fragment DNA content defined better response and prognosis (22).

### Viral HPV ctDNA

As a substantial proportion of SCHNCs of the oral cavity and oropharynx are HPV-related tumors (23), quantification of HPV ctDNA in liquid biopsies is useful to detect remnant HPV+ tumors and eventually monitor the efficacy of RT/CRT. An interesting study by Damerla *et al.* examined 97 patients with HPV+ oropharyngeal cancer (OPC), where detectable HPV16 ctDNA was observed in 90/97 cases, and HPV33 ctDNA in additionally three cases. A study by Veyer *et al.* analyzed 66 patients with HPV+ OPC and showed that high HPV ctDNA levels were linked with advanced T- and N-stage (24).

Leung *et al.* applied NGS for HPV-sequencing in patients with OPC treated with CRT and demonstrated that detectable HPV ctDNA at the end of treatment was linked with poor progression-free survival (PFS) (25). Cao *et al.* examined 34 cases with stage III OPC treated with CRT (26). Low pretreatment levels and an early rise in HPV ctDNA during the first two weeks of therapy was associated with better PFS. In a report by Chera *et al.* on 67 patients with HPV+ OPC, HPV16 ctDNA levels directly correlated with tumor burden (27). More than 95% clearance of HPV16 ctDNA was obtained in 19/67 of patients on the 28<sup>th</sup> day of CRT, and none of these patients had residual disease or relapsed after CRT. In contrast, 35% of the rest of patients recurred after therapy. A large study by Dahlstrom *et al.* included 262 OPC patients, reporting that pretreatment HPV ctDNA was higher in patients with node involvement and distant metastasis (28). Although authors did not specify the treatment of patients, undetectable HPV ctDNA before therapy among patients with HPV+ tumors, was linked with better PFS.

### Viral EBV ctDNA

EBV is also a causative factor for the development of non-keratinizing and undifferentiated nasopharyngeal cancer (NPC), especially in North Africa and Asia (29). EBNA1 and latent membrane proteins LMP1 and LMP2 are encoded by the EBV viral genome and can be targeted for ctDNA-assessment in liquid biopsies.

Lo *et al.* studied the EBV ctDNA in the plasma of 389 NPC patients treated with RT (30). During the follow-up, 63 patients developed metastasis, and 60 of them had detectable EBV ctDNA. In 23 out of 45 patients who developed local relapse, EBV ctDNA was also detectable. Moreover, Li *et al.*

assessed the pre-RT plasma levels of EBV ctDNA (BamHI-W region of the virus), reporting detectable levels in 69.4% of patients (31). Locoregional recurrence was linked with persistent EBV ctDNA detection in 56.4% of patients during the follow-up, while this rose to 93.9% in patients who developed metastasis. Detectable EBV ctDNA was noted in only 12.8% of patients who remained without disease. A study by Wang *et al.* reported on 41 NPC patients who underwent PET-CT after RT, showing that all 36 patients with detectable EBV (BamHI-W region) ctDNA post-treatment had recurrent tumor, while five patients with undetectable EBV ctDNA levels had normal PET-CT imaging (32).

A large study on 1,984 patients with non-metastatic NPC has been published by Chen *et al.* (33), where assessment of EBV ctDNA was serially performed during the follow-up of patients after RT. Detectable EBV ctDNA (BamHI-W region) was recorded in 767 (38.7%) of patients and 63.8% of them developed disease relapse, whereas patients with undetectable EBV ctDNA had a lower relapse rate (8.6%). Detection of ctDNA preceded by a median of 2.3 months (up to 9.5 months) the clinical manifestation of tumor.

### Mutated ctDNA

SCHNC is frequently linked with mutations of genes related to cell proliferation and survival (*TP53*, *EGFR*, *HRAS*, *RET*, and *PIK3CA*), cellular differentiation (*NOTCH1*, Hedgehog, *WNT*), cell adhesion (*FAT1,4*), and cell-cycle regulation [cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin D1 (*CCND1*)]. Panels of such genes have been used to detect mutations of ctDNA in liquid biopsies (34-36). van Ginkel *et al.* reported a retrospective analysis of 239 patients with SCHNC showing that *TP53* mutations are detectable in 83% of tumor samples, while other gene mutations like in *CDKN2A*, *PIK3CA*, *HRAS*, *CDK4*, *FBXW7* and *RBI* genes ranged from 0-21% (37). Similarly, Flach *et al.* found that *TP53* mutations were the most frequently detected in ctDNA analysis, followed by *NOTCH1*, *NF1* and *CDKN2A* gene mutations (38). Detection of *TP53* mutations in ctDNA is quite high, as Economopoulou *et al.* reported a rate of 32.6% in a series of 45 SCHNC patients, a percentage close to the 40% recorded in cancer tissue samples from the same patients (39). In addition, Wang *et al.* documented ctDNA somatic mutations in the saliva of 76% of SCHNC patients examined (34). Besides HPV ctDNA, authors found *TP53* mutations in 92% of samples. However, there are no reports in the literature focusing on detecting mutations in ctDNA and response to RT/CRT. The LIONESS study was designed to detect up to 52 somatic variants in 17 patients with SCHNC treated with surgery (40). During the follow-up, ctDNA mutations preceded clinical documentation of disease progression by 10-253 days.

Focusing on ESCC, Eyck *et al.* reported a study on 24 cases of ESCC with confirmed mutations in bioptic tissue material, where detection of mutations in ctDNA before treatment was feasible in 38% of them (41). Wang *et al.* reported a study on 40 patients treated with RT/CRT, showing that out of 16 patients who had progressive disease after therapy 69% had detectable ctDNA at a median of 4.4 months before radiological detection (42). Detectable ctDNA one month or 3-6 months after therapy was linked with inferior PFS, while ctDNA clearance within one month from therapy defined a better prognosis. Azad *et al.* published a series of 45 ESCC patients who underwent preoperative CRT (43). Detectable ctDNA after CRT was linked with shorter PFS, and detection preceded radiological confirmation of progression by an average of 2.8 months. Of interest, several patients had new mutations detected in the ctDNA after CRT.

Morimoto *et al.* found that in ESCC patients treated with neoadjuvant chemotherapy, persistent ctDNA after surgery related with poor PFS (recurrences 90% vs. 0%) (44). Low post/pre-chemotherapy ctDNA mutation levels have been linked with better overall survival in a series of 42 ESCC patients (45). High ctDNA levels in patients with ESCC have been also linked with poor response to immunotherapy with immune checkpoint inhibitors (46).

### Methylated ctDNA

Gene hypo- and hyper-methylation is an epigenetic mechanism of repression or over-expression of genes. The best-studied methylation occurs in the 5-carbon cytosine position in CpG dinucleotides. Biron *et al.* reported differential histone gene methylation between HPV+ and negative OPCs (47, 48). Hypermethylation of *WT1*, *PAX6*, *CADMI*, *RARβ* genes in HPV+ oropharyngeal cancer has been documented, and *PAX5* methylation occurred mainly in metastatic disease (49).

The methylation status of ctDNA can also be assessed in liquid biopsies from patients with SCHNC (50). In a study by Birkenova *et al.*, ctDNA methylation was detectable years before the clinical manifestation of cancer (51). CpG site methylation of promoter regions of 807 genes has been reported in the saliva of patients with SCHNC (52), and arrays have been proposed as biomarkers.

A study by Righini *et al.* in 90 patients with SCHNC examined the methylation status of 11 genes, showing that at least one of the genes *MGMT*, *p16*, *RASSF1A*, and *p16* was methylated in 75% of patients, but this was not related to the prognosis of patients. Nevertheless, abnormal methylation was detected during the follow-up of patients a few months before clinical documentation of relapse (53). However, the study did not specify the treatment type that patients had received.

Table I. *Studies on circulating tumor (ctDNA) assessment in the plasma of cancer patients with squamous cell head neck cancer (SCHNC) and esophageal squamous cell carcinoma (ESCC), and its relation with treatment outcome.*

Author/(year)/(reference)	No pts	Disease	Treatment	Main findings
<b>Non-specific ctDNA</b>				
Mazurek <i>et al.</i> (2016) (18)	200	SCHNC	Not-stated	Correlation with advanced stage and lymph node metastasis
Verma <i>et al.</i> (2020) (19)	24	SCHNC	CRT	Increasing ctDNA levels after CRT was related with residual disease
Kim <i>et al.</i> (2021) (20)	61	ESCC	CRT	Short fragment ctDNA content was related with better response and prognosis
<b>Viral HPV ctDNA</b>				
Veyer <i>et al.</i> (2020) (22)	66	HPV+ OPC	Not stated	High HPV ctDNA was linked with advanced T-stage and lymph node involvement
Leung <i>et al.</i> (2021) (23)	13	HPV+ OPC	CRT	Detectable HPV ctDNA at the end of treatment was linked with poor PFS
Cao <i>et al.</i> (2022) (24)	34	Stage III OPC	CRT	Low pretreatment HPV ctDNA and early rise during the first 2 weeks of CRT were linked with better PFS
Chera <i>et al.</i> (2019) (25)	67	HPV+ OPC	CRT	More than 95% HPV ctDNA clearance after CRT was noted in 28% of patients, and was linked with tumor eradication
Dahlstrom <i>et al.</i> (2015) (26)	262	OPC	Not stated	High HPV ctDNA levels were linked with node involvement and distant metastasis. Undetectable HPV ctDNA before therapy was linked with better PFS
<b>Viral EBV ctDNA</b>				
Lo <i>et al.</i> (1999) (28)	389	NPC	RT	Detectable EBV ctDNA was observed in 60/63 patients who developed metastasis, and in 23/45 who had local relapse
Li <i>et al.</i> (2017) (29)	385	NPC	RT	Persistent EBV ctDNA after RT was linked Locoregional recurrence and metastasis
Wang <i>et al.</i> (2011) (30)	41	NPC	RT	Patients with detectable EBV ctDNA after RT had recurrent tumor
Chen <i>et al.</i> (2020) (31)	1984	NPC	RT	Detectable EBV ctDNA after RT was linked with 63.8% relapse vs. 8.6% of patients who had undetectable EBV ctDNA. Detection of ctDNA preceded by a median of 2.3 months the radiological documentation of relapse
<b>Mutated ctDNA</b>				
Wang <i>et al.</i> (2015) (32), van Ginkel <i>et al.</i> (2016) (35), Flach <i>et al.</i> (2022) (36), Economopoulou <i>et al.</i> (2023) (37)		SCHNC	Not stated	Although several studies report high rates of mutations detection in the plasma and saliva, there are no data regarding their prognostic or predictive relevance after RT/CRT
Flach <i>et al.</i> (2022) (38)	17	SCHNC	Surgery	Detection of ctDNA mutations preceded documentation of tumor progression by 10-253 days
Wang <i>et al.</i> (2022) (40)	40	ESCC	CRT	In 69% of patients with recurrent disease, ctDNA was detectable by a median of 4.4 months before radiological documentation. ctDNA clearance within one month after RT was linked with better PFS
Azad <i>et al.</i> (2020) (41)	45	ESCC	Neoadjuvant CRT	Persistent ctDNA detection after surgery was related with poor PFS. ctDNA detection preceded radiological confirmation of progression by an average of 2.8 months
Morimoto <i>et al.</i> (2021) (42)	13	ESCC	Neoadjuvant chemotherapy	Persistent detection of ctDNA after surgery was related with poor PFS
Fujisawa <i>et al.</i> (2021) (43)	42	ESCC	Neoadjuvant chemotherapy	Low levels of ctDNA before and after chemotherapy defined better prognosis
Chen <i>et al.</i> (2023) (44)	30	ESCC	Neoadjuvant ICIs and chemotherapy	High ctDNA levels defined poor prognosis
<b>Methylated ctDNA</b>				
Righini <i>et al.</i> (2007) (51)	90	SCHNC	Not stated	Abnormal methylation of ctDNA was detected months before documentation of relapse
Rettori <i>et al.</i> (2013) (52)	200	SCHNC	Not stated	TIMP3 hypermethylation was related with better prognosis
Shen <i>et al.</i> (2020) (53)	53	OPC	CRT	Hypermethylation of EDNRB gene preceded the detection of relapse after RT
<b>TMB ctDNA</b>				
Wildsmith <i>et al.</i> (2023) (54)		SCHNC	ICIs	High TMB ctDNA was linked with better response to ICIs

OPC: Oropharyngeal cancer; NPC: nasopharyngeal cancer; CRT: chemo-radiotherapy; RT: radiotherapy; PFS: progression-free survival; ICIs: immune checkpoint inhibitors.

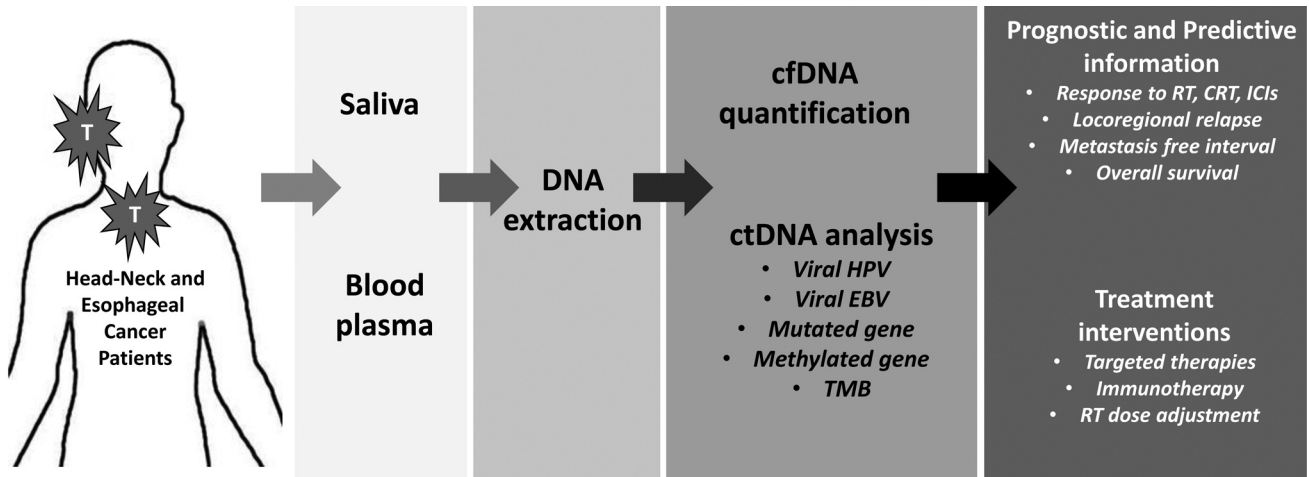


Figure 1. Schematic representation of liquid biopsies, type of analysis, and eventual clinical value in patients with squamous cell head-neck and esophageal cancer T: Tumor; RT: radiotherapy; CRT: chemo-radiotherapy; ICI: immune checkpoint inhibitor; TMB: tumor mutational burden.

Another study by Rettori *et al.* examined the methylation status of genes in the saliva of patients with SCHNC, identifying five hypermethylated genes with high specificity for the presence of tumor, namely *CCNA1*, *DAPK*, *DCC*, *MGMT* and *TIMP3* (54). Hypermethylation of the *TIMP3* was linked with better local-recurrence free survival, but authors did not comment on the type of treatment offered to patients. Shen *et al.* also reported on 53 patients with OPC treated with CRT, where hypermethylation of the *EDNRB* gene as detected in the saliva of patients preceded the detection of clinical relapse after therapy (55).

### Tumor Mutational Burden ctDNA

The tumor mutational burden (TMB) assessed in cancer tissues has been established as a standard biomarker for the administration of immune checkpoint inhibitors (ICIs). Assessment of TMB by NGS provides the total number of mutations existing in the tumor genome. High TMB has been associated with better overall survival (OS) in SCHNC patients treated with immunotherapy (56, 57). It is postulated that the higher number of mutations relates to a higher number of mutated proteins produced by the tumor, leading to a high number of antigenic peptide release and presentation by HLAs, increasing tumor antigenicity and recognition by dendritic and cytotoxic T-cells.

TMB assessment is also feasible in plasma samples from ctDNA, although inconsistencies have been reported among the various existing blood-NGS tests (58). Wildsmith *et al.* reported that TMB in ctDNA was not related to PD-L1 expression or HPV-status in SCHNC but was associated with better PFS and OS in patients treated

with durvalumab (anti-PD-L1) and tremelimumab (anti-CTLA4) (56). Combination of RT with avelumab anti-PD-L1 immunotherapy failed to show a benefit when added to standard CRT (59). Similarly, the French GORTEC 2015-01 reported a lack of benefit from the addition of pembrolizumab to RT vs. cetuximab-RT (60). The use of ctDNA-based TMB assessment may prove useful to identify subgroups of SCHNC patients who would benefit from the addition of ICIs to standard RT and CRT.

### Conclusion

Liquid biopsies and detection of ctDNA in the plasma and saliva emerge as important tools to integrate in the diagnostic and therapeutic algorithm for patients with SCHNC and ESCC. Table I reports the published studies and main findings of liquid biopsy analysis in patients with SCHNC and ESCC. Initial circulating viral (HPV or EBV) ctDNA levels, and levels of total, mutated or methylated ctDNA are linked with tumor burden and clinical aggressiveness, and may be of prognostic or even predictive value of RT/CRT efficacy. Persistent ctDNA levels after therapy seem to predict high rates of tumor relapse several months before radiological documentation. This can prove of value for the identification of subgroups of patients who could benefit from RT dose-escalation or consolidation chemotherapy and immunotherapy, a hypothesis that should be tested in clinical trials (Figure 1).

### Conflicts of Interest

There are no conflicts of interest to report in relation to this study.

## Authors' Contributions

IMK: Literature search, analysis, writing of the first draft, approval for submission; EX: Literature search, analysis, writing of the first draft, approval for submission; MIK: Conception, design, critical analysis of papers, writing, approval for submission.

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