Circulating tumor cells (CTCs) are transient cancer cells which have the capacity to metastasize to distant sites such as the lungs and liver in HNSCC. When metastatic disease is radiographically evident, the patient prognosis is often poor. Therefore, methodologies to assess micrometastatic disease are needed to (1) identify patients likely to develop metastatic disease and (2) treat and monitor these patients more aggressively. Whilst CTCs are well documented in other tumor streams such as breast, colorectal cancer and prostate cancers, the data and clinical utility in HNSCC remains limited. Areas covered: Here we summarize the recent advances of CTCs and applications in HNSCC.

Expert opinion: CTC enumeration can be prognostic in HNSCC; further studies are warranted to investigate the role of CTC clusters in HNSCC; CTC culture (in vivo/ex vivo) may present a possibility to expand these rare cells to a critical mass for functional testing; PD-L1 expression of HNSCC CTCs may present a means by which to determine patients likely to respond to therapy; a HNSCC CTC-specific marker is warranted.

1. Introduction

Head and neck squamous cell cancers (HNSCCs) represent the 7th most common malignancy globally. Whilst historically the disease has been associated with alcohol and smoking, of recent there has been an increase in human papillomavirus (HPV) associated HNSCCs, particularly HPV-16 induced oropharyngeal cancers [1]. Patients often present with locally advanced disease where a combined modality of treatment is given including combinations of surgery, radiotherapy and chemotherapy [2]. These therapies do offer a degree a local control, however distant metastatic disease often results in poor patient outcomes. Immune checkpoint inhibitors (ICI) that block the PD-1/PD-L1 pathway have demonstrated un paralleled response (20%-30%) in the metastatic setting, with clinical trials data showing an improved progression free survival and overall survival compared to the standard arm of therapy [3–5]. Furthermore, the USFDA has recently also approved Pembrolizumab as a first-line treatment for patients with advanced HNSCC based on the pivotal Phase 3 KEYNOTE-048 trial [5]. ICI treatment however is cost intensive and associated with potential immune related toxicities, therefore identifying patients likely to respond is paramount. There remains a need for biomarkers which are able to select the most suitable patient for this highly targeted immunotherapy [6,7].

Liquid biopsies have come to the fore due to sampling being noninvasive, can be repeated easily and sampled over the course of therapy [8,9]. Liquid biopsy approach includes biomarkers such as CTCs, cell-free DNA (including circulating tumor DNA; ctDNA) and exosomes. ctDNA has shown clinical utility in recapitulating the molecular profile of the tumor [10–13] and has recently been used to derive blood tumor mutation burden (bTMB) metrics which have been shown to be predictive biomarkers of response to immunotherapy [14,15].

Circulating tumor cells (CTCs) represent transient metastatic precursor cells with the propensity to metastasize to distant sites. CTCs were first described by Thomas Ashworth in 1869 who described cells in the blood of a man which were identical to the tumor itself [16]. Since this discovery, CTC clusters or aggregates of CTCs were also reported by Watanabe in 1954 [17]. In breast, prostate and colorectal cancer, CTC enumeration has demonstrated to be prognostic using the FDA-approved CellSearch platform (Menarini Silicon Biosystems, Italy), where CTC enumeration has correlated with progression free survival and overall survival [18,19]. This technology relies on tumor cells expressing an epithelial marker for preselection (EpCAM). EpCAM has been shown to vary significantly in HNSCCs due to epithelial-mesenchymal transition (EMT) and therefore has poor capture efficiencies using this technology [20]. Alternative technologies using epitope-independent CTC capture have been explored for HNSCCs successfully [2,20–22]. To this end, a number of studies have demonstrated a higher CTC capture efficiency using label-free technologies [21,23,24]. However, the clinical significance of
Circulating tumour cells have shown promise as liquid biopsies in advanced stage head and neck cancers. Circulating tumour cell threshold/cut-off values need to be established for head and neck cancers. Beyond enumeration alone, the data on circulating tumour cell clusters, and circulating tumour microemboli is emerging in head and neck cancers. Single cell circulating tumour cell characterization is giving insights into CTC heterogeneity. Expansion of head and neck circulating tumour cells may allow for functional analysis. Immunotherapeutic biomarkers (e.g. PD-L1) have been identified on single cell circulating tumour cells.

EpCAM-negative CTCs is yet to be ascertained. Head-to-head studies comparing the CellSearch platform with other epitope independent technologies [21] is warranted in order to understand the role of CTC populations at play which may correspond with clinical outcomes. A limitation in the field has been the functional characterization of CTCs, which has become more attainable using ex vivo and in vivo culture methodologies to expand these rare cancer cells [25–28]. The functional analysis of CTCs has the potential to reveal novel therapeutic approaches to target metastatic disease [29,30]. More recently, and in part due to PD-L1 being non-predictive in the tumor tissue, alternative sources of tumor material which may provide a comprehensive overview of a patient’s PD-L1 status has been sought for which CTCs have shown some early promise [7,31,32].

2. CTC characterization

2.1. CTC enumeration

CTC counts and cutoffs from CellSearch (Menarini Silicon Biosystems) were derived for metastatic breast (mBC), prostate (mPC) and colorectal cancer (mCRC) where ≥ 5 CTCs were associated with unfavorable outcomes in mBC and mPC and ≥ 3 CTCs in mCRC [19]. Threshold values have not been established for HNSCC by the CellSearch technology. A number of groups have investigated the presence of CTCs in HNSCC using a number of CTC enrichment technologies including microfluidics, microfiltration, ISET, CellSearch, negative depletion, density gradient centrifugation, immunomagnetic beads, laser scanning cytometry, flow cytometry and RT-PCR [33]. Comparison across CTC enrichment platforms and the resulting CTC populations can be challenging as each technology may enrich for a different population of CTCs. Therefore, matched patient samples and head-to-head comparisons with the CellSearch will be ultimately needed to determine the CTC population that is being captured and whether it is prognostic [21]. To further compound this, the molecular analysis of CTCs has shown that there is a vast heterogeneity in CTCs at the single cell level [34]. Therefore, it is critical to understand the metastatic propensity of individual CTCs. This has become possible using single-cell CTC approaches which capture single CTCs either manually/automatically and undergo whole genome amplification and next generation sequencing [35]. Studies have shown that single CTCs can be classified into 3 types: Type 1 which shares genomic copy number changes with the CTCs and tumor; Type 2 where copy number changes were detected but not detected in the tumor and Type 3, where no copy number changes were detected [36]. Therefore, the role of each sub category needs to be elucidated to understand the heterogeneity found in CTC subtypes. This gives an additional layer of complexity into CTC biology and simple enumeration of CTCs may overestimate the actual number of CTCs with a propensity to metastasize. Other confounding factors include the sheer forces in the blood which could result in poor viability of CTCs [2]. Studies have shown that single CTCs have a longer half-life in blood compared to CTC clusters and therefore are more likely to face the stressors found in the blood [2].

2.2. CTC cluster studies and microemboli

CTC clusters represent 3 or more CTCs which move through the lymphovascular collectively. These heterogeneous multicellular aggregates are commonly termed CTC clusters. Circulating Tumor Microemboli (CTM) or circulating micrometastasis [37–39]. Numerous groups have reported that CTC clusters appear to have a higher metastatic capacity than single cells for seeding at distant sites, evidenced by their short transit time in blood, ability to form micrometastasis at favorable sites more readily than single cells and ability to traverse through narrow capillaries in a single-file and reform the cluster upon exiting the narrow capillaries [38,40–43]. In breast cancer, it has been shown that CTC clusters have a 23–50 times higher metastatic capacity compared to single cells [38]. CTC clusters have shown to express gamma-catenin (plakoglobin), which is a major cytoplasmic component of desmosomes and adherens junctions. In a recent study by Aceto and colleagues, it was found that in breast cancer, binding sites for stemness and proliferation associated transcription factors (e.g. OCT4, NANO, SOX2, SIN3A) were hypomethylated in CTC clusters. Moreover, this study suggested that cluster targeting compounds may be used to reduce the effects of cluster mediated metastasis in breast cancer patients [44]. Additionally, CTC clusters have been associated with WBCs. In particular, neutrophils have been found to promote efficient metastasis formation by releasing cytokines which drive cell cycle progression within the bloodstream as well as protecting CTCs in circulation. In the study, when the release of cytokines was blocked, this reduced the pro-metastatic effects of neutrophils within CTC clusters [43]. In the study by Murlidhar et al., 2017 in early stage lung cancer, it was shown that CTC clusters were characteristic of therapeutic resistance highlighting the aggressive nature of these cellular aggregates [45]. CTC clusters have been reported in HNSCC using a number of technologies including spiral and straight microfluidic technologies using epitope-independent CTC capture [46,47]. In our recent study, we have demonstrated that CTC clusters in HNSCC patients contain EGFR-amplified CTCs of comparable sizes to CTMs [47]. EGFR is amplified in HNSCC tumor tissues, thus confirming the likelihood of HNC origin [48]. Other common alterations in HNSCC include FGFR1, MYC, A. KULASINGHE ET AL.
CCND1 and PIK3CA, all of which have not yet been investigated in HNSCC CTCs.

3. CTC expansion

3.1. CTC ex vivo expansion

There have been a number of research groups that have demonstrated CTC expansion for functional studies and the creation of CTC cell lines in lung, prostate and colon cancer using stem-like culture media [28,49–51]. Notably, in these studies, CTC cultures were attainable from patients with high tumor burden/volume of disease where their immediate prognosis was poor [52,53]. Therefore, whilst it is desirable to expand CTCs to understand the biology and functional role of these cells, it remains a challenge where long-term cultures can only be generated in advanced/late stage patients. Conversely, a number of groups have shown that CTCs, including CTC clusters can be expanded in the short-term where drug sensitivity testing may be possible [50,54]. The Nagarath group has demonstrated that in early stage lung cancer, CTCs can be expanded using microfluidic co-culture models including fibroblasts and extracellular matrix (ECM). In our previous study, we were able to demonstrate short-term expansion of CTCs from advanced stage HNSCC patients under hypoxic conditions. Of the samples where cultures were attainable, 71.4% originated from an HPV-positive patient cohort. We hypothesized that the HPV oncogenes E6 and E7 may have a role to play in inducing a replicative senescent state. A recent study by Liao and colleagues has demonstrated in HNSCC that CTC spheroid culture could serve as a model to mimic tumor formation during metastasis [55]. Moreover, the study demonstrated an epithelial (E-type) and mesenchymal (M-type) CTC phenotype with the M-CTC count higher in patients with distant metastasis (p = 0.066).

3.2. In vivo expansion of CTC

The Dive group has demonstrated that patient derived explant (PDX) models can be generated in small cell lung cancer and that in some cases, in vivo response mirrors patient responses in clinic to platinum agents [56,57]. Yu et al., demonstrated that CTC-cultures were attainable in 6 estrogen receptor positive breast cancer cases. Of which, three of a total of five CTC lines were tumorigenic in mice. Genotyping of the CTC cell lines identified subsets of mutations that had therapeutic targets likely to benefit the patient. Therefore, single or drug combinations, including standard clinical treatment options were tested against the CTC cell lines. The study demonstrated that standard therapy, including tamoxifen, raloxifene and fulvestrant were ineffective in one CTC cell line. Additionally, in an another CTC cell line (BRx-07), that de novo mutations were acquired over the course of therapy in PIK3CA and FGFR2 and that targeting these mutations showed cooperative effect [51].

3.3. Diagnostic leukapheresis

Diagnostic Leukapheresis (DLA) is a routine procedure based on continuous centrifugation which is used to enrich mononuclear cells from blood (1.055–1.08g/ml), a cell population which has similar densities to CTCs [58]. Studies have shown that by using larger blood volumes, a larger proportion of CTCs is able to be captured for downstream analysis and ex vivo expansion of patient derived CTCs [59,60]. In fact, recent studies have shown that processing of DLA product results in a 0–32 fold increase in CTC capture compared to the standard 7.5ml blood analysis [61]. As HNSCC typically presents with relatively low numbers of CTCs (e.g. <20 CTCs/7.5ml blood) [2], DLA may represent a methodology by which to assess larger blood volumes, which may in turn result in higher CTC numbers for downstream analysis/expansion studies.

4. Immunotherapeutic biomarkers

ICI have been hailed as paradigm changing in cancer therapeutics and is now considered a pillar of cancer treatment [3]. However, there remains a need to better identify patients that would be likely to respond to ICI treatment. In primary tumor tissue, PD-1/PD-L1 has been shown to be non-predictive of outcome to ICI treatment as PD-1/PD-L1 are dynamic markers which cannot be sampled by a static snapshot (e.g. tumor biopsy). To this end, CTCs have been proposed as candidate biomarkers to identify PD-L1 status as CTCs represent cancer cells from both primary and metastatic sites in real time. The first study demonstrating that CTCs frequently expressed PD-L1 was shown in breast cancer [32]. Since this finding, numerous groups have investigated the role of PD-L1 in CTCs [62–65]. In a landmark study, Strati et al., 2017 developed a PD-L1 mRNA assay which could determine PD-L1 expression in HNSCC CTCs by RT-qPCR. Notably, in this study, PD-L1 was found to be overexpressed at baseline in 25.5% of HNSCC patients, 23.5% after induction chemotherapy and 22.2% after treatment. The presence of PD-L1 overexpressing CTCs after treatment associated with a shorter progression free survival (P = 0.001) and overall survival (p < 0.001). Moreover, the study found that the absence of PD-L1 overexpression at the completion of treatment correlated with a complete response [31]. In the recent study by Chikamatsu and colleagues, they identified three immune-regulatory molecules (CD47, PD-L1 and PD-L2) which were correlated with each other in 24 HNSCC CTC positive patients. In this study, the PD-L1 expression in tumor tissue did not correspond to that of CTCs [65]. We have published a recent case report where CTCs and CTC clusters were identified in an advanced stage HNSCC patient that expressed PD-L1. Furthermore, when the PD-L1 was quantified, it was found to be highly expressed [64]. In a longitudinal follow up study, we found that in HNSCC patients, CTC-positivity associated with a shorter progression free survival (PFS) (P = 0.0063) than in patients with the absence of CTCs and the PD-L1 positivity in CTCs was found to be significant (P = 0.0485). These studies warrant the investigation of PD-L1 in HNSCC CTCs to determine whether it can be used as a companion diagnostic test to determine whether patients would respond to ICI therapy based on CTC findings [7].
5. Universal CTC marker

Whilst it has been desirable to have a single CTC marker characteristic of CTCs, this has been a complex issue due to several reasons. The CellSearch definition of a CTC is that of a cell which is EpCAM, cytokeratin (8, 18, 19), DAPI positive and CD45 negative. As EpCAM is used for pre-selection, the CellSearch technology has demonstrated that EpCAM positive CTCs are of clinical significance [18]. To this end, a number of studies has shown the importance of EpCAM positive CTCs [66] including applications of PD-L1+ CTCs in clinical studies [67,68]. However, there have been reports of CTCs expressing dynamic levels of epithelial and mesenchymal markers in circulation [39,69]. These findings support the notion that to become a CTC, cancer cells typically undergo an epithelial-mesenchymal transition to become more aggressive and invasive, allowing cells to break through the basement membranes and into the lymphovascularature. Moreover, studies have shown that EMT-CTCs or mesenchymal CTCs are found in patients with progressive disease post treatment highlighting a possible mechanism of treatment resistance. In HNSCC, due to the variable and low expression of EpCAM, it has been desirable to use alternative markers to capture and characterize CTCs. A number of studies in HNSCC have utilized the overexpression of EGFR as this is associated with a high proportion of HNSCC tumor tissue [70]. A recent HNSCC study by Economopoulou et al., 2019 demonstrated a highly sensitive RT-qPCR assay for HPV16 E6/E7 mRNA expression in EpCAM-positive CTCs [71]. In the study, a significantly higher risk of relapse (p = 0.001) and death (0.005) was associated with HPV16 E6/E7 baseline CTCs demonstrating that this may be used to define subsets of HNSCC patients.

To overcome the complexities with marker based CTC capture, a number of CTC enrichment technologies have been developed for epitope independent CTC capture using parameters such as size, deformability, density and electric properties to name a few [72,73]. This allows for the capture of CTCs without a pre-selection marker bias. In turn, allowing for the characterization of a greater proportion of CTC populations. A number of studies have shown that ‘small-CTCs’ do exist of comparable sizes to normal blood cells which may not be captured using size based CTC capture [66]. Therefore, complimentary or a number of CTC enrichment platforms may be needed to capture the majority of CTCs in circulation. In some cases, ‘no enrichment’ has been the preferred methodology for CTC capture. Laboratories such as the Kuhn lab and Epic Sciences which use a ‘no-cell left behind technology’ allow for the profiling of the total cellular pellet from blood. No enrichment platforms have the added benefit of characterizing the total nucleated fraction of cells in circulation. The high definition single-cell analysis (HD-SCA) workflow combines the entire population of CTCs and rare cancer cells with single-cell genomic analysis through whole-genome amplification, library preparation to copy number variation (CNV) analysis of identified cells [34]. These studies have begun to reveal the multiple CTC populations in blood and the heterogeneity that is present at a single time point and longitudinally over the course of therapy [74–76].

6. Conclusion

This review article summarizes the current state-of-play in CTCs with regard to HNSCC and methodologies which have been trialed in other tumor types and could be applied to HNSCC. The CTC findings in HNSCC could be used to compliment conventional imaging modalities to report on micrometastatic disease. An example of this is the recent inclusion in breast cancer TNM (tumor, node, metastasis) staging. In patients with cMo(i+), there is no sign of cancer upon physical examination/scans/x-rays, but cancer cells are present in the blood/bone marrow/lymph nodes from blood analysis. Furthermore, longitudinal CTC studies need to be performed in HNSCC to understand how CTC phenotypes change over time/during therapy. Moreover, if CTCs can be expanded during the course of therapy via a blood draw, this may provide opportunities for functional testing of CTCs outside the patient’s body.

7. Expert opinion

Repeat tumor biopsies to determine treatment efficacy are often challenging to perform and have associated risks for the patient. Therefore, alternative, less invasive means of assessing tumoral information are needed. Liquid biopsies may address this unmet clinical need and have come to the fore in profiling tumoral information noninvasively over the course of treatment. Liquid biopsies enable serial sampling, which can be performed multiple times during treatment to determine changes in tumoral information in real time. In addition, after the completion of treatment, liquid biopsies can be used to determine minimal residual disease prior to radiographic progression. In so doing, allowing for a window of treatment opportunity before the development of metastatic disease. With the emergence of novel CTC enrichment methodologies (e.g. microfluidic, size based filtration, acoustic, magnetic beads, antibody based), there remains a need to benchmark the findings to the FDA-approved CellSearch platform to determine the role of the CTC populations captured by these alternative technologies and to determine their relevance in disease progression/prognosis. In addition to CTC clusters, it is important to determine the role of other circulating populations: tumor associated macrophages (TAMs), circulating endothelial cells (CECs), circulating stem cells (CSCs) and their role in disease progression. CTC expansion outside the patient’s body may allow for functional CTC analysis to be performed. The functional properties of CTCs needs to be investigated in order to understand how these cells move from the primary tumor into the lymphovascularature, exist in the blood, and extravasate to distant sites. CTCs expressing immunotherapeutic biomarkers may be used to identify patients likely to respond to targeted therapy (e.g. Immunotherapies). As CTCs are shed from primary and metastatic sites, they are likely to be more representative of the disease as a whole compared to a single site biopsy.

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References
Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Chai RC, Lambie D, Verma M, et al. Current trends in the etiology and diagnosis of HPV-related head and neck cancers. Cancer Med. 2015 Apr;4(4):596–607. PubMed PMID: 25644715; PubMed Central PMCID: PMCPMC4402074. eng.
2. Kulasinghe A, Perry C, Jovanovic L, et al. Circulating tumour cells in metastatic head and neck cancers. Int J Cancer. 2015 Jun 01;136(11):2515–2523. PubMed PMID: 25111594; eng.
3. Ferris RL, Blumschein G Jr., Fayette J, et al. Nivolumab for recurrent squamous-cell carcinoma of the head and neck. N Engl J Med. 2016 Nov 10;375(19):1856–1867. PubMed PMID: 27718784; PubMed Central PMCID: PMCPMC5564292. eng

• Key immunotherapy clinical trial in head and neck squamous cell carcinoma

4. Cohen EEW, Soulières D, Le Tourneau C, et al. Pembrolizumab versus methotrexate, docetaxel, or cetuximab for recurrent or metastatic head-and-neck squamous cell carcinoma (KEYNOTE-040): a randomised, open-label, phase 3 study. Lancet. 2019 Jan 12;393(10167):156–167.
5. Rischin D, Harrington KJ, Greil R, et al. Protocol-specified final analysis of the phase 3 KEYNOTE-048 trial of pembrolizumab (pembro) as first-line therapy for recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC). J Clin Oncol. 2019 May 20;37(15_suppl):6000.
6. Maleki Vareki S, Garrigós C, Durán I. Biomarkers of response to PD-1/PD-L1 inhibition. Crit Rev Oncol Hematol. 2017 Aug 01;116:116–124.
7. Kulasinghe A, Kenny L, Punyadeera C. Circulating tumour cell PD-L1 test for head and neck cancers. Oral Oncol. 2017 Dec;75:6–7. PubMed PMID: 29224824; eng.
8. Aix-Panabieres C, Pantel K. Clinical applications of circulating tumour cells and circulating tumor DNA as liquid biopsy. Cancer Discov. 2016 May;6(5):479–491. PubMed PMID: 26969689; eng.
9. Bardelli A, Pantel K. Liquid biopsies: What We Do Not Know (Yet). Cancer Cell. 2017 Feb;31(2):172–179.
10. Abbosh C, Birkbak NJ, Wilson GA, et al. Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. Nature. 2017 Apr 26;545(7655):446–451. PubMed PMID: 28445469; eng.
11. Schmidt H, Kulasinghe A, Kenny L, et al. The development of a liquid biopsy for head and neck cancers. Oral Oncol. 2016 Oct;61:18–11. PubMed PMID: 27688098; eng.
12. Schmidt H, Kulasinghe A, Allcock RN, et al. A pilot study to non-invasively track PIK3CA mutation in head and neck cancer. Diagnostics (Basel). 2018 Nov 29;8(4). PubMed PMID: 30501041; PubMed Central PMCID: PMCPMC6315660. eng.
13. Schmidt H, Kulasinghe A, Perry C, et al. A liquid biopsy for head and neck cancers. Expert Rev Mol Diagn. 2016;16(2):165–172. PubMed PMID: 26631411; eng.
14. Hofman P, Heeke S, Aix-Panabieres C, et al. Liquid biopsy in the era of immune-oncology. Is it ready for prime-time use for cancer patients? Ann Oncol. 2019 Jun 22 PubMed PMID: 31228184; eng. 10.1093/annonc/mdz196.
15. Wang Z, Duan J, Cai S, et al. Assessment of blood tumor mutational burden as a potential biomarker for immunotherapy in patients with non-small cell lung cancer with use of a next-generation sequencing cancer gene panel. JAMA Oncol. 2019 Feb 28 PubMed PMID: 30816954; PubMed Central PMCID: PMCPMC6512308. eng. 10.1001/jamaoncol.2018.7098.
16. Ashworth T. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. Australas Med J. 1869:14:146–147.
17. Watanabe S. The metastasizability of tumor cells. Cancer. 1954 Mar;2(2):215–223. PubMed PMID: 13141212; eng.
18. Allard WJ, Matera J, Miller MC, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res Off J Am Assoc Cancer Res. 2004 Oct 15;10(20):6897–6904. PubMed PMID: 15501967; eng.
19. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumour cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004 Aug 19;351(8):781–791. PubMed PMID: 15317891; eng.
20. Kulasinghe APC, Boyle GM, O’Byrne K, et al. Epithelial-mesenchymal axis in head and neck cancer cell lines. J Solid Tumors. 2016;6:1.
21. Kulasinghe A, Kenny L, Perry C, et al. Impact of label-free technologies in head and neck cancer circulating tumour cells. Oncotarget. 2016 Nov 17;7(44):71223–71234. PubMed PMID: 27655722; PubMed Central PMCID: PMCPMC5342074. eng

• Head-to-head comparative study across multiple CTC enrichment technologies for head and neck cancer.

22. Perumal V, Corica T, Dharmarajan AM, et al. Circulating tumour cells (CTC), head and neck cancer and radiotherapy; future perspectives. Cancers (Basel). 2019 Mar 15;11(3). PubMed PMID: 30875950; PubMed Central PMCID: PMCPMC6468366. eng.
23. McMullen KP, Chalmers JJ, Lang JC, et al. Circulating tumour cells in head and neck cancer: A review. World J Otorhinolaryngol Head Neck Surg. 2016 Jun;2(2):109–116. PubMed PMID: 29204555; PubMed Central PMCID: PMCPMC5698518. eng.
24. Kulasinghe ATT, Blick T, O’Byrne K, et al. Enrichment of circulating head and neck tumour cells using spiral microfluidic technology. Scientific Reports. 2017;7(press).
25. Aix-Panabieres C, Pantel K. Liquid biopsy in cancer patients: advances in capturing viable CTCs for functional studies using the EPISPOT assay. PubMed PMID: 26390240; eng Expert Rev Mol Diagn. 2015;15(11):1411–1417.
26. Cayrefourcq L, Mazard T, Josse S, et al. Establishment and characterization of a cell line from human circulating colon cancer cells. Cancer Res. 2015 Mar 1;75(3):892–901. PubMed PMID: 25592149; eng.

• An important paper describing long term CTC culture establishment

27. Laloo A, Gulati S, Schenk MW, et al. Ex vivo culture of cells derived from circulating tumour cell xenograft to support small cell lung cancer research and experimental therapeutics. Br J Pharmacol. 2019 Feb;176(3):436–450. PubMed PMID: 30427531; PubMed Central PMCID: PMCPMC6329630. eng.
28. Gao D, Vela I, Sboner A. Organoid cultures derived from patients with non-small cell lung cancer with use of a next-generation sequencing cancer gene panel. JAMA Oncol. 2019 Jun 22 PubMed PMID: 31228184; eng. 10.1093/annonc/mdz196.
29. Drone C, Brady G. SnapShot: circulating tumour cells. Cell. 2017 Feb 9;168(4):742–742.e1. PubMed PMID: 28187292; eng.
30. Strati A, Koutsodontis G, Papaioinou G, et al. Prognostic significance of PD-L1 expression on circulating tumour cells in patients with head and neck squamous cell carcinoma. Ann Oncol. 2017 Aug 01;28(8):1923–1933. PubMed PMID: 28838214; eng.
• Characterisation of PD-L1 in HNC CTCs
32. Mazel M, Jacot W, Pantel K, et al. Frequent expression of PD-L1 on circulating breast cancer cells. Mol Oncol. 2015 Nov;9(9):1773–1782. PubMed PMID: 26093818; eng.
33. Perumal V, Corica T, Dharmarajan AM, et al. Circulating tumour cells (CTC), head and neck cancer and radiotherapy; future perspectives. Cancers (Basel). 2019;11(3):367. PubMed PMID: 30875950; eng.
34. Thiele JA, Pitule P, Hicks J, et al. Single-cell analysis of circulating tumour cells. Methods Mol Biol. 2019;1908:243–264. PubMed PMID: 30649733; eng.
35. Zhu Z, Qiu S, Shao K, et al. Progress and challenges of sequencing and analyzing circulating tumor cells. Cell Biol Toxicol. 2018;34(5):405–415. PubMed PMID: 29168077; eng.
36. Chemi F, Gulati S, Rothwell DG, et al. Abstract 5601: single-cell molecular profiling of circulating tumor cells (CTCs) within the TRACERx study reveals heterogeneous patterns in early non-small cell lung cancer (NSCLC). Cancer Res. 2018;78(13 Supplement):5601. AM2018-5601.
37. Umer M, Vaidyanathan R, Nguyen NT, et al. Circulating tumor microemboli: progress in molecular understanding and enrichment technologies. Biotechnol Adv. 2018 Jul - Aug;36(4):1367–1389. PubMed PMID: 29753882; eng.
38. Aceto N, Bardia A, Miyamoto DT, et al. Circulating tumor cell clusters are oligoocular precursors of breast cancer metastasis. Cell. 2014 Aug 28;158(5):1110–1122. PubMed PMID: 25171411; PubMed Central PMCID: PMCPMC4149753; eng.
39. Yu M, Bardia A, Wittner BS, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science (New York, NY). 2013 Feb 1;339(6119):580–584. PubMed PMID: 23372014; PubMed Central PMCID: PMCPMC3760262; eng.
• Important paper describing the EMT nature of CTCs
40. Au SH, Storey BD, Moore JC, et al. Clusters of circulating tumor cells traverse capillary-sized vessels. Proc Natl Acad Sci U S A. 2016 May 3;113(18):4947–4952. PubMed PMID: 27091969; PubMed Central PMCID: PMCPMC4983862; eng.
• CTC cluster study demonstrating that CTC clusters retain their cluster forming capacity when travelling through narrow capillaries.
41. Krol I, Castro-Giner F, Maurer M, et al. Detection of circulating tumour cell clusters in human glioblastoma. Br J Cancer. 2018 Aug 1 PubMed PMID: 30065256; eng. doi: 10.1038/s41416-018-0186-7.
42. Sarioglu AF, Aceto N, Kojic N, et al. A microfluidic device for label-free physical capture of circulating tumor cell clusters. Nat Methods. 2015;12(7):685–691. PubMed PMID: 25984697; PubMed Central PMCID: PMCPMC4490017; eng.
43. Szczepanek M, Castro-Giner F. Neutrophils escort circulating tumour cells to enable cell cycle progression. Nature. 2019 Feb 6;566(7745):553–557. doi: 10.1038/s41586-019-0915-y. PubMed PMID: 30728496; eng.
44. Gkoutselas S, Castro-Giner F, Szczepanek M, et al. Circulating tumor cell clustering shapes DNA methylation to enable metastasis seeding. Cell. 2019 Jan 10;176(1–2):98–112.e14. PubMed PMID: 30633592; PubMed Central PMCID: PMCPMC6363966. eng.
45. Muralidhar V, Reddy RM, Fouladdel S, et al. Poor prognosis indicated by venous circulating tumor cell clusters in early-stage lung cancers. Cancer Res. 2017;77(18):5194–5206. PubMed PMID: 28716896; eng.
46. Kulasinghe A, Schmidt H, Perry C, et al. A collective route to head and neck cancer metastasis. Sci Rep. 2018 Jan 15;8(1):746. PubMed PMID: 29335441; eng
• One of the first studies demonstrating CTC clusters in HNC.
47. Kulasinghe A, Zhou J, Kenny L, et al. Capture of circulating tumour cell clusters using straight microfluidic chips. Cancers (Basel). 2019 Jan 14;11(1). PubMed PMID: 30646614; eng.
• Study demonstrating EGFR amplification in HNC CTC clusters
48. TCGA. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015 Jan 29;517(7536):576–592. PubMed PMID: 25631445; PubMed Central PMCID: PMCPMC4311405; eng.
49. Soler A, Carefouroglu L, Mazard T, et al. Autologous cell lines from circulating colon cancer cells captured from sequential liquid biopsies as model to study therapy-driven tumor changes. Sci Rep. 2018 Oct 28;9(1):15931. 10.1038/s41598-018-34365-z. PubMed PMID: 30374140; PubMed Central PMCID: PMCPMC6206901. eng.
50. Khoo BL, Greci G, Lim YB, et al. Expansion of patient-derived circulating tumor cells from liquid biopsies using a CTC microfluidic culture device. Nat Protoc. 2018 Jan;13(1):34–58. PubMed PMID: 29215634; eng.
51. Yu M, Bardia A, Aceto N, et al. Cancer therapy. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. Science (New York, NY). 2014 Jul 11;345(6193):216–220. PubMed PMID: 25031076; PubMed Central PMCID: PMCPMC4358808. eng.
52. Kapeliers J, Kulasinghe A, Warkiani ME, et al. The prognostic role of circulating tumor cells (CTCs) in lung cancer. Front Oncol. 2018;8:311. PubMed PMID: 30155443; PubMed Central PMCID: PMCPMC6102369. eng.
53. Kulasinghe A, Perry C, Warkiani ME, et al. Short term ex-vivo expansion of circulating head and neck tumor cells. Oncotarget. 2018;9(27):27517751; Eng Aug 9 2016; 10.18632/oncotarget.11159
• First manuscript describing short term CTC culture in HNC
54. Khoo BL, Greci G, Jing T, et al. Liquid biopsy and therapeutic response: circulating tumor cell cultures for evaluation of anticancer treatment. Sci Adv. 2016 Jul;2(7):e1600274. PubMed PMID: 27453941; PubMed Central PMCID: PMCPMC4956185. eng.
55. Liao CJ, Hsieh CH, Hung FC, et al. The integration of a three-dimensional spheroid cell culture operation in a circulating tumor cell (CTC) isolation and purification process: a preliminary study of the clinical significance and prognostic role of the CTCs isolated from the blood samples of head and neck cancer patients. Cancers (Basel). 2019 Jun 6;11(6). PubMed PMID: 31174311; eng.
56. Carter L, Rothwell DG, Mesquita B, et al. Molecular analysis of circulating tumor cells identifies distinct copy-number profiles in patients with chemosensitive and chemoresistant small-cell lung cancer. Nat Med. 2017 Jan 23(1):114–119. PubMed PMID: 27869802; eng.
57. Hodgkinson CL, Morrow CJ, Li Y. Tumorigenicity and genetic profiling of circulating tumor cells in small-cell lung cancer. Nat Med. 2014 Aug;20(8):897–903. doi: 10.1038/nm.3734. PubMed PMID: 24880617; eng.
58. Fehm TN, Meier-Stiegen F, Driemel C, et al. Diagnostic leukapheresis for CTC analysis in breast cancer patients: CTC frequency, clinical experiences and recommendations for standardized reporting. Cytometry A. 2018 Dec;93(12):1213–1219. PubMed PMID: 30551262; eng.
59. Fischer JC, Niederacher D, Topp SA, et al. Diagnostic leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer patients. Proc Natl Acad Sci U S A. 2013 Oct 8;110(41):16580–16585. PubMed PMID: 24065821; PubMed Central PMCID: PMCPMC3799344. eng.
60. Franken A, Driemel C, Behrens B, et al. Label-free enrichment and molecular characterization of viable circulating tumor cells from diagnostic leukapheresis products. Clin Chem. 2019 Apr;65(4):549–558. PubMed PMID: 30737205; eng.
61. Andree KC, Mentink A, Zeune LL, et al. Toward a real liquid biopsy molecular characterization of viable circulating tumor cells from liquid biopsies using a CTC microfluidic trap: CTCTrap. Int J Cancer. 2018 Nov 15;143(10):2584–2591. PubMed PMID: 30006930; eng.
62. Kulasinghe A, Kapeliers J, Kimberley R, et al. The prognostic significance of circulating tumor cells in head and neck and non-small-cell lung cancer. Cancer Med. 2018 Dec 7:5910–5919. PubMed PMID: 30565869; PubMed Central PMCID: PMCPMC6308060. doi: 10.1002/cam4.1832
63. Kallergi G, Vetiska EK, Aggououri D, et al. Evaluation of PD-L1/CD274 on circulating tumor cells in patients with advanced non-small cell lung cancer. Ther Adv Med Oncol. 2018;10:1758834017750121.
64. Kulasinghe A, Perry C, Kenny L, et al. PD-L1 expressing circulating tumour cells in head and neck cancers. BMC Cancer. 2017 May 16;17(1):333. PubMed PMID: 28511705; PubMed Central PMCID: PMCPMC5434641. eng.
65. Chikamatsu K, Tada H, Takahashi H, et al. Expression of immune-regulatory molecules in circulating tumor cells derived from patients with head and neck squamous cell carcinoma. Oral Oncol. 2019;89:34–39.
66. Fachin F, Spuhler P, Martel-Foley JM, et al. Monolithic chip for high-throughput blood cell depletion to sort rare circulating tumor cells. Sci Rep. 2017 Sep 07;7(1):10936. PubMed PMID: 28883519; PubMed Central PMCID: PMCPMC5389885. eng.
67. Janning M, Kobus F, Babayan A, et al. Determination of PD-L1 expression in circulating tumor cells of NSCLC patients and correlation with response to PD-1/PD-L1 inhibitors. Cancers (Basel). 2019 Jun;11(6). PubMed PMID: 31212989; eng.
68. Nicolazzo C, Raimondi C, Mancini M, et al. Monitoring PD-L1 positive circulating tumor cells in non-small cell lung cancer patients treated with the PD-1 inhibitor Nivolumab. Sci Rep. 2016 Aug 24;6:31726. PubMed PMID: 27553175; PubMed Central PMCID: PMCPMC5995431. eng.
69. Liu X, Li J, Cadilha BL, et al. Epithelial-type systemic breast carcinoma cells with a restricted mesenchymal transition are a major source of metastasis. Sci Adv. 2019 Jun;5(6):eaav4275. PubMed PMID: 31232646; PubMed Central PMCID: PMCPMC6584608. eng.
70. The Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015 Jan 29;517(7536):576–582. doi: 10.1038/nature14129. PubMed PMID: 25631445; PubMed Central PMCID: PMCPMC4311405. eng.
71. Economopoulou P, Koutsodontis G, Averis M, et al. HPV16 E6/E7 expression in circulating tumor cells in oropharyngeal squamous cell cancers: A pilot study. PLoS One. 2019;14(5):e0215984. PubMed PMID: 31071126; PubMed Central PMCID: PMCPMC6508656. eng.
72. Kulasinghe A, Wu H, Punyadeera C, et al. the use of microfluidic technology for cancer applications and liquid biopsy. Micromachines. 2018 Aug 10;9(8). PubMed PMID: 30424330; PubMed Central PMCID: PMCPMC6187606. eng.
73. Bailey PC, Martin SS. Insights on CTC biology and clinical impact emerging from advances in capture technology. Cells. 2019;8(6):553.
74. Gerdtsson E, Pore M, Thiele JA, et al. Multiplex protein detection on circulating tumor cells from liquid biopsies using imaging mass cytometry. Converg Sci Phys Oncol. 2018 Mar;4(1). PubMed PMID: 30906572; PubMed Central PMCID: PMCPMC6430142. eng. doi:10.1088/2057-1739/aaa013.
75. Nieva J, Wendel M, Luttgen MS, et al. High-definition imaging of circulating tumor cells and associated cellular events in non-small cell lung cancer patients: a longitudinal analysis. Phys Biol. 2012 Feb;9(1):016004. PubMed PMID: 22306961; PubMed Central PMCID: PMCPMC3388002. eng.
76. Dago AE, Stepansky A, Carlsson A, et al. Rapid phenotypic and genomic change in response to therapeutic pressure in prostate cancer inferred by high content analysis of single circulating tumor cells. PLoS One. 2014;9(8):e101777. PubMed PMID: 25084170; PubMed Central PMCID: PMCPMC4118839. eng.
