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REVIEW ARTICLE

Circulating tumor cells in head and neck cancer: A review

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Abstract Carcinoma of the head and neck represents 3.5% of all cancers, and the vast majority of these tumors are squamous cell carcinoma (HNSCC). With a stable overall survival rate of 50% among all stages, there is continued interested in developing measures for early detection and disease aggressiveness. Circulating tumor cells (CTCs) have been identified as a potential marker for early metastatic disease, response to treatment, and surveillance in head and neck squamous cell carcinoma. In this article, techniques of CTC detection, applications of CTC technology, and outcomes of HNSCC patients will be discussed.
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Introduction

Cancer of the upper aerodigestive system comprised an estimated 59,340 cases in the United States in 2015 and 3.5% of all cancers,[1] with squamous cell carcinoma of the head and neck (HNSCC) making up approximately 95% of these tumors. The overall 5-year survival for all stages combined is approximately 50%, and this overall figure has not changed much in the past few decades. Although there have been advances in disease stratification according to site (HPV association with improved survival in

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oropharyngeal carcinoma[2,3]), there is a relative paucity of information with regards to predicting outcome in HPV negative oropharyngeal tumors and those from other aerodigestive sites. Presence of lymphatic metastases is a main indicator of poor prognosis; however, microscopic metastases to lymph nodes occur in 11%–50% of head and neck tumors, depending on site.[4–6] Metastases develop when tumor cells acquire properties to invade the local lymphatic and vascular spaces, migrate into the bloodstream or lymphatic system, and then develop the ability to proliferate in lymph nodes and distant sites. The capacity to measure tumor cells circulating through the vasculature provide definitive evidence of the aggressiveness of a tumor prior to detection of identifiable metastases. If a blood test existed, this may improve the ability to stratify patients for a particular treatment. Circulating tumor cells (CTCs) have been identified as a marker for disease severity in other fields, with poor outcomes in breast,[7] colorectal,[8,9] and prostate cancer,[10,11] among others. In head and neck oncology, identification of circulating tumor cells in patients with squamous cell carcinoma may be a promising tool toward development of a "liquid biopsy" for disease severity, providing early and definitive evidence of metastatic disease. Detection of CTCs may also provide opportunities for targeted treatment through genetic analysis, aid in response to treatment, and serve as a marker for post-treatment surveillance. Over the past twenty years, there have been significant advances in the field of circulating tumor cells in SCCHN.

CTC detection methods

Detecting CTCs in peripheral blood is a challenge by virtue of the seven to eight order of magnitude difference in the number of normal circulating blood cells and abnormal tumor cells in circulation. In each milliliter of whole blood, there are approximately 5 billion red blood cells, 295 million platelets, and 7 million white blood cells.[12] Identification methods have evolved over the past two decades and now focus on surface markers of intact cells. Although CTCs are a heterogeneous population, they differ from normal circulating peripheral blood cells — it is through these differences that they are extracted from a blood sample.

Early methods for CTC identification utilized RT-PCR for detection of tumor-specific antigens, assuming that these antigens were not found in normal circulating blood.[13–15] One of the first studies in head and neck cancer with regard to CTCs looked at cytokeratin 20, an epithelial marker that is expressed in many squamous carcinomas. In blood samples of patients with oral squamous cell carcinoma, there was an association between the presence of mRNA for cytokeratin 20 and a reduced disease free survival and increased lymph node metastases.[16] However, a major limitation to identification of CTCs utilizing this technique is the lack of visual confirmation of the tumor cell. Although RT-PCR is an efficient and reproducible technique, false positives from non-viable cellular material or from amplification of non-specific genomic material cannot be excluded.

Most labs currently utilize techniques that can directly visualize the cells of interest. Using positive selection methods, epithelial surface antigens are targeted on the surface of CTCs and "pulled out" of the blood sample. EpCAM (epithelial cell adhesion molecule) is one of the most commonly utilized markers and is the primary antigen used in the only FDA-approved circulating tumor cell detection equipment, CellSearch (Janssen Diagnostics, NJ, USA). CellSearch utilizes a semi-automated process that identifies EpCAM positive CTCs and "illuminates" them for identification using fluorescently labeled cytokeratin 8, 18, and 19. While this method is effective for identification of EpCAM positive cells, it has become apparent in recent years that metastatic cells display significant plasticity and may lose EpCAM expression, thereby evading this detection method.[17–19] Konisberg and colleagues detected EpCAM negative CTCs in metastatic breast cancer which were not picked up by EpCAM dependent enrichment methods.[17] This raises the concern of false negatives if an EpCAM-only method is utilized. Another well-studied marker for CTC identification is cytokeratin (CK) and in the authors' experience, this has proven to be more sensitive in detection of CTCs. Within the cytokeratin family, CK 8, 18, 19, and 20 have been targeted the most in head and neck CTCs. However, even cytokeratin may not be present on all circulating tumor cells. The downregulation of EpCAM, and to a lesser extent cytokeratin, is thought to be related to epithelial to mesenchymal transition (EMT), whereby the tumor cell loses its epithelial identity. Recent studies have demonstrated this CTC heterogeneity: Weller et al noted subpopulations of HNSCC CTCs with absence of CK, but presence of N-cadherin (mesenchymal origin) and CD133 (stem-cell origin).[20] Balsubramanian et al also detected CTCs with presence of N-cadherin and Vimentin (mesenchymal origin), suggesting de-differentiation of a subset of tumor cells that may elude standard identification techniques.[21] These findings highlight the importance of an unbiased approach to CTC detection using multi-marker testing. It also appears that the studies utilizing CellSearch identify a much lower absolute number of CTCs. CellSearch appears to identify numbers in the single digits[22–24] more frequently than those utilizing other detection methods, which may reach thousands.[25,26] Because there is no normative value of CTCs in the bloodstream, it is difficulty to draw conclusions as to which method is optimum; however, it would make sense that methods utilizing positive selection may "miss" CTCs because of low or nonexistent surface markers that are being targeted.

Contrary to the positive selection techniques above, negative depletion techniques, such as the one utilized by the authors, aim to remove as many "normal" blood cells as possible in order to relatively increase the number of circulating tumor cells in a sample. Using this method, an average of 5.66 log_{10} enrichment is achieved, which allows subsequent identification and characterization of CTCs without relying on a particular biomarker. The tumor cells can then undergo multi-marker staining; the presence of a greater reduction in number of cells facilitates the manual identification of the cells. In the first step of this process, red blood cells (which make up the majority of circulating blood cells) are removed by lysis. Leukocytes are then labeled with anti-CD45 and removed by immunomagnetic separation.[27] With a majority of normal nucleated blood cells removed, the remaining cells undergo nuclear staining with DAPI (4′,6-diamidino-2-phenylindole) and further

immunocytochemical staining. Based on this information, the definition of a circulating tumor cell is a nucleated (DAPI+) intact cell, negative for anti-CD45 and positive for cytokeratin or other identified epithelial or mesenchymal markers. Fig. 1 displays an example of confocal microscopy and immunostaining characteristics of circulating tumor cells using this approach.

Blood is typically drawn from of a peripheral venous source, distant to the site of malignancy/intervention. In an effort to reduce the chance of contamination, it has been generally accepted, but not verified, that the first few mL from the peripheral stick should be discarded. A majority of outcomes studies have examined CTCs measured this way, although one study recently looked at measuring CTCs from drain aspirates after extirpation for locally advanced cN0 head and neck squamous cell carcinoma and adenocarcinoma. They detected between 3 and 2094 CTCs/ml in 64% patients using the CellSearch method, although the clinical significance is still unknown.[28] One of the limitations of the immunocytochemical tests is the variability in CTC recovery rates. As noted before, the rarity of these cells in peripheral blood is one of the main challenges to overcome. Hristozova and colleagues noted a mean recovery rate of 69% in CTC spiked control samples using their detection method.[29] Reithdorf and colleagues examined the inter and intra- assay variability as well as the stability of the cell line over time using the CellSearch method. Overall, they found reasonable reliability and reproducibility. Samples could be processed up to 72 h after blood draw with good reliability. However, when looking at controls spiked with a specified number of CTCs, the recovery rates using CellSearch produced a mean of 80% (range: 30%—100%), indicating that there are limitations to enumerating all of the known CTCs in a sample. They did not, however, have a "negative" sample in the spiked controls, demonstrating that CTCs were always found in the sample even if the number was less than expected.[30]

The field of microfluidics has been proposed to present a number of advantages in the recovery of CTCs and has been evolving over a number of years. By way of a recent microfluidic "chip assay" method, a volume of fluid is applied to a very sensitive and specific "chip," which acts as a micro screen for detecting the CTC through DNA or RNA aptamers. Early studies have shown this approach to be a highly sensitive and specific tool.[31] Using temperature sensitive aptamers, it has been demonstrated that tumor cells can be released in a viable state for further analysis. It is also reusable, allowing for sensitivity >90% with repeated use up to 6 times. The authors also report potential improved cost with this method (owing to the reusability of the equipment), although a direct analysis was not done.

Clinical applications

With the knowledge of CTCs evolving in the field of head and neck surgery, there is interest in determining what role it plays in the treatment of head neck cancer. Detection of CTCs in HNSCC range from 16% to 80% depending on the detection method utilized.[14,23—26,29,32—34] One of the most promising ideas in circulating tumor cell detection in head and neck squamous cell carcinoma is the correlation with patient survival. Table 1 illustrates HNSCC CTC studies examining survival and response to treatment. A majority of studies have noted reduced disease free survival,[25,26,34] reduced progression free survival, and overall survival.[32] Follow up for these studies is notably short, ranging 19—36 months. Another study has noted no difference in survival, although follow-up was only six months.[22] A recent meta-analysis examining circulating tumor cells in SCCHN revealed that patients with CTCs had a significantly increased risk of tumor progression, although presence of CTCs did not appear to be related to T or N stage.[37] A predominating theory behind the differences seen in these studies is the heterogeneity of CTCs, and that not every tumor cell will go on to form a metastasis, or are related to a metastasis. Efforts in the future should be targeted to identifying the subset or subpopulation of cells that display these aggressive properties.

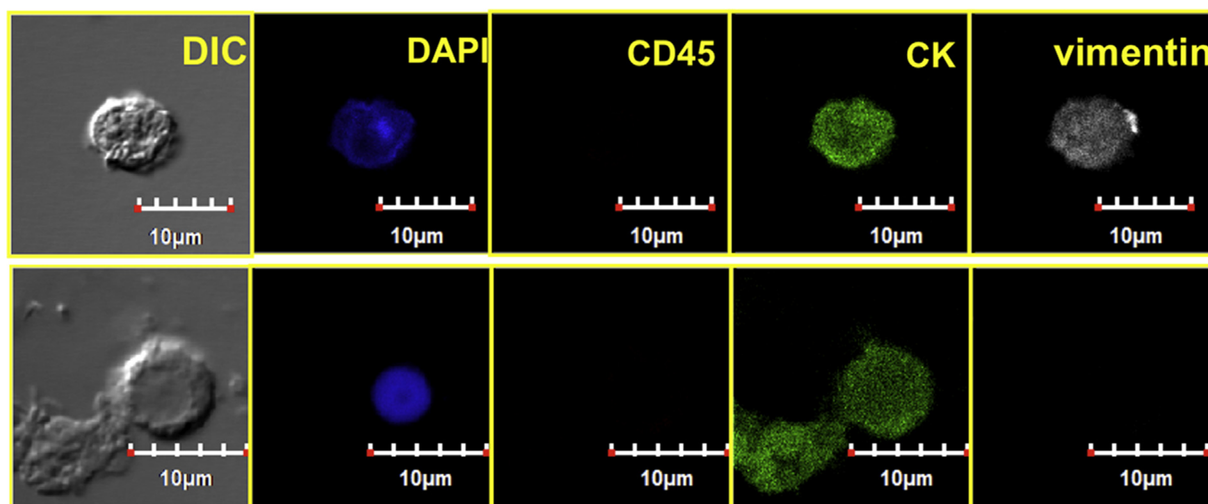

Fig. 1 Multi-marker staining of circulating tumor cells in SCCHN. A CTC (DIC) with corresponding immunocytochemical marker analysis. As defined, the cells are DAPI+ (4′,6-diamidino-2-phenylindole) and CD 45−. Both are positive for cytokeratin (CK); however, the first is also positive for mesenchymal marker, vimentin.

Table 1 Summary of head and neck CTC studies.

Study	N	Study design	Treatment population (stage)	Method of detection	When CTCs measured	Range CTCs	Marker	% With CTCs	Mean Follow-up, months	Outcome (+)CTC: (−) CTC	Other outcomes
Wirtshafter et al (2002)[14]	18	Prospective	I–IV	Positive Selection ICC	Before treatment	0–3	EpCAM	44%	N/A	Outcomes not examined	
Partridge et al (2003)[34]	36	Prospective	I–IV	Negative Depletion ICC and RT-PCR	Before, after surgery	0–5	Pan-CK, E48	50%	36	Reduced DFS	Poor agreement between RT-PCR and ICC
Winter et al (2009)[35]	16	Prospective	I–IV	RT-PCR	Before, after surgery	N/A	EGFR, CK, ELF3, Eph84	63%	N/A	No difference	
Jatana et al (2010)[26]	48	Prospective	I–IV	Negative Depletion ICC	Before treatment	0–3300	CK	71%	19	Reduced DFS	
Hristozova et al (2011)[29]	42	Prospective	LR advanced	Flow cytometry and RT-PCR	Before treatment	0–4	EpCAM, CK	43%	N/A	Assoc with ≥N2b disease	Decrease in CTC with chemoradiation
Nichols et al (2011)[24]	15	Prospective	III–IV	CellSearch (Positive Selection ICC)	Before treatment	0–2	EpCAM	40%	N/A	CTC presence assoc. with lung nodules >1 cm; reduced survival	
Buglione et al (2012)[23]	73	Prospective	HNSCC and SNUC (I–IV)	CellSearch (Positive Selection ICC)	Before, during, after treatment	0–43	EpCAM	15%	14	No difference	Decrease in CTC over treatment correlated with response; CTCs presence trended positively with increasing stage (NS)
Bozec et al (2013)[22]	49	Prospective	III–IV	CellSearch (Positive Selection ICC)	N/A	0–5	EpCAM	16%	6	No difference	
Hseih et al (2014)[33]	53	Prospective	LR advanced, recurrent, metastatic	Negative Depletion ICC	Before treatment	N/A	EpCAM	19%	10.5	PDPN+/EpCAM + ratio >20% prognostic factor for DSM	
Grisanti et al (2014)[32]	53	Prospective	Recurrent/ Metastatic	CellSearch (Positive Selection ICC)	Before, during, after treatment	0–43	EpCAM	26% baseline; 41% at any point	25	Reduced PFS and OS	

Tinhofer et al (2014)[36]	144	Prospective	III–IV	RT-PCR	After surgery, before adjuvant	N/A	EpCAM, CK	29%	34	Reduced DFS and OS in Non-OPSCCa; opposite effect in OPSCC	No assoc. between CTC presence and HPV in OPSCCa
Inhestern et al (2015)[25]	40	Prospective	II–IV	Flow cytometry	Before, during, after treatment	0–11298	EpCAM	80% baseline; 97% at any point	23	Baseline CTCs > median = reduced RFS and Maximal CTCs at any point > median = reduced OS	Overall CTCs decreased during IC; increased after surgery; decreased after radiotherapy

ICC: immunocytochemistry; EpCAM: epithelial cell adhesion molecule; RT-PCR: reverse transcriptase – polymerase chain reaction; CK: cytokeratin; DFS: disease free survival; EGFR: epidermal growth factor receptor; LR: locoregional; CTC: circulating tumor cell; HNSCC: head and neck squamous cell carcinoma; SNUC: sinonasal undifferentiated carcinoma; NS: not significant; PDPN: podoplanin; OS: overall survival; OPSCC: oropharyngeal squamous cell carcinoma.

One biomarker of interest in head and neck CTCs is podoplanin. Recent studies have examined podoplanin (PDPN), a trans-membrane protein involved in lymphatic formation, as a possible predictor of poor outcomes in head and neck squamous cell carcinoma. Retrospective studies have associated the degree of PDPN expression with decreased survival in cutaneous squamous cell carcinoma[38] and oral squamous cell carcinoma,[39,40] although one study suggests no association.[41] All studies measured presence and degree of expression of PDPN via immunohistochemical techniques. It appears however, that PDPN may be lost during de-differentiation in squamous carcinoma.[41] Therefore, lack of PDPN may not guarantee an improved survival. Continuing this line of investigation, Hseih et al[33] looked at PDPN presence in CTCs of patient with locally advanced and metastatic HNSCC and found that when the percentage of an individual's CTC count contained >20% PDPN+/EpCAM + cells, there was a significant independent prognostic factor for poor progression free and overall survival at 12 months. Absolute presence of CTCs did not have an effect on outcomes in this study, which supports the notion of cell subtypes driving the outcomes.

Oliveira-Costa et al[42] performed whole genome sequencing of oral cavity SCCa patients using microarray analysis in an effort to identify additional biomarkers that portend a worse prognosis. They found that PDL-1 (programmed death ligand) expression was upregulated in the primary tumor as tumor size increased. They then examined the association of circulating tumor cells with PDL-1 and disease specific survival in a separate cohort of patients. Interestingly, they found an improved short-term survival benefit with the presence of cytoplasmic PDL-1 on CTCs. This was an unexpected finding given previous literature suggesting association between PDL-1 and progression of disease. However, in recent years, other fields have noted a similar finding between PDL-1 upregulation and improved survival: in colorectal cancer,[43] Merkel cell carcinoma,[44] and metastatic melanoma.[45] Other preliminary work has been done by measuring PDL-1 and its receptor, PD-1, in oral cavity SCCa,[46,47] but no additional conclusions regarding outcomes could be surmised. With PD-1 and PDL-1 being the target of Phase I and II therapies in other fields of oncology, more research and knowledge can be expected in the future.

EGFR has also been suggested as a possible CTC biomarker for disease aggression in HNSCC, given its association with poor response to treatment in head and neck cancer.[48] Grisanti and colleagues measured CTCs in recurrent and metastatic HNSCC. They detected 26% and 41% of CTCs at baseline and any point during treatment, respectively.[32] Within that study, 45% of the patients had EGFR expression on CTCs. An interesting finding was that, in patients with multiple detected CTCs, only 25% of tumor cells expressed EGFR, indicating evidence of CTC heterogeneity within each tumor. No outcome analysis was done comparing EGFR positivity in this study. In another study, Tinhofer and colleagues also measured EGFR and its phosphorylated form, pEGFR in patients with locoregionally advanced HNSCC.[49] In their patient population, 100% of sampled were +EGFR, and 55% were +pEGFR. When looking at their two treatment groups, which included induction chemotherapy followed by radiotherapy plus cetuximab vs.

concurrent chemoradiotherapy, they found that the percentage of pEGFR expressing CTCs decreased more in the Cextuximab group than in the concurrent group. In later follow-ups, however, it appeared that the percentage of pEGFR positive CTCs began to rise again in the Cetuximab group. This is an expected finding given that Cetuximab targets EGFR, and highlights the possibility of measuring clinical response to targeted therapy with serial CTC measurement.

The optimal time or interval to measure CTCs is not yet clear. Many studies in head and neck measure CTCs at only one point during an intervention (typically prior), although studies have shown that CTCs fluctuate over the course of treatment. Inhestern and colleagues recently published their results examining changes in circulating tumor cells in patients with oral and oropharyngeal SCCa.[25] For all patients, this trial included induction chemotherapy followed by stratification based on response with subsequent surgery/postoperative therapy. They found that response to induction chemotherapy was the most significant predictor of overall survival. Patients who had a baseline CTC measurement greater than the median baseline of the group (3925 CTCs/ml) had significantly worse disease-free survival than those with baseline numbers less than the median. Overall survival was also lower in individuals whose maximal CTC count at any point was higher than the median maximal CTC count (5005 CTCs/ml). They also found that CTCs fluctuated during the course of treatment: generally, tumor cells decreased during chemotherapy, increased after surgery, and decreased after radiation. Interestingly, 80% of patients were found to have CTCs at the beginning of treatment, and this number increased to 97% at the conclusion of treatment.

There is developing research with regards to treatment related fluctuations of CTCs and whether this has a prognostic effect. Kusakawa and colleagues detected cytokeratin using RT-PCR shortly after incisional biopsy of patients with oral SCCa but not in their controls or patients who underwent excisional biopsy.[36] This was one of the first suggestions in head and neck that direct manipulation within a tumor may release CTCs in the vasculature. Similar findings have been noted in primary lung cancer resection as well.[50]

Jurati et al[51] have done work with regard to *in vivo* monitoring of CTCs in murine melanoma and breast cancer models. Using fluorescence flow cytometry by monitoring a single peripheral vein, they have identified dynamic changes in the number of CTCs when the tumor is manipulated. In the breast cancer model, there was no difference in CTC dynamics when compared to controls with simple pressure, analogous to mammography or pressure/manipulation during surgery. In the punch biopsy group, the number of CTCs increased 82% immediately and remained elevated for five weeks after. On the contrary, complete surgical resection (including negative margins) resulted in a decrease in tumor cells, although tumor cells were again detected at very low levels greater than 5 weeks after surgery despite absence of any clinical recurrence. These findings again suggest that tumor disruption may lead to an increase in CTCs.

A central question from these studies is whether release of CTCs by tumor manipulation affects prognosis. These cells are artificially released into the circulation. As a result, it is unclear whether these "surgically-released" tumor cells have acquired the phenotype capable of causing metastasis. This makes sense clinically, as a tumor may be actively manipulated during extirpation with no consequence.

A chief limitation to research has been the heterogeneous detection methods by which laboratories detect CTCs. A single, reproducible method of CTC identification would standardize research at a multi-institutional level. As discussed previously, CellSearch meets criteria for this unifying test, but is not ideal given its reliance on EpCAM. Although negative depletion and chip assay techniques are unbiased, a standardized and marketable platform has not been established yet. As methods for detecting CTCs are further refined, we can expect an improved test in the future that satisfies all the components above.

Conclusion

Detection and characterization of circulating tumor cells is a promising tool for identification of early metastatic disease, response to treatment, and surveillance in head and neck squamous cell carcinoma. It has been shown in multiple studies that presence of CTCs pre-treatment indicates a decreased survival in the short term with long term studies underway.

As detection methods evolve, it will be important to identify which subpopulations of CTCs play a role in tumor aggressiveness. As a result, an unbiased, multi-marker approach to detection is necessary in order to identify the heterogeneous population of cells. Standardization of detection protocols would also aid in unifying the clinical recommendations that can be made for influencing treatment decisions.

Conflict of interest

The authors declare no conflict of interest related to this work.

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