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REVIEW

Squamous cell carcinoma of the oral cavity and circulating tumour cells

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Abstract

Due to a lack of substantial improvement in the outcome of patients suffering from oral squamous cell carcinoma (OSCC) during the past decades, current staging methods need to be revised. This disease is associated with poor survival rates despite considerable advances in diagnosis and treatment. The early detection of metastases is an important indicator of survival, prognosis and relapse. Therefore, a better understanding of the mechanisms underlying metastasis is crucial. Exploring alternative measures apart from common procedures is needed to identify new prognostic markers. Similar to previous findings predominantly for other solid tumours, recently published studies demonstrate that circulating tumour cells (CTCs) and disseminated tumour cells (DTCs) might serve as prognostic markers and could supplement routine staging in OSCC. Thus, the detection of CTCs/DTCs is a promising tool to determine the individual need for therapeutic intervention. Encouraging results and new approaches point to the future use of targeted therapies for OSCC, an exceedingly heterogeneous subgroup of head and neck cancer. This review focuses on summarising technologies currently used to detect CTCs/DTCs. The translational relevance for OSCC is highlighted. The inherent challenges in detecting CTCs/DTCs will be emphasised.

Key words: Circulating tumour cells; Disseminated tumour cells; Oral squamous cell carcinoma; Head and neck squamous cell carcinoma; Bone marrow; Peripheral blood; Micrometastasis; Minimal residual disease; Epithelial-mesenchymal transition

Core tip: Oral squamous cell carcinoma (OSCC), among head and neck cancer, is related to poor survival rates despite considerable advances in diagnosis and treatment. Therefore, detecting tumour cell dissemination early and understanding the underlying mechanisms are crucial for predicting prognosis, relapse and survival. According to previous findings, circulating tumour cells (CTCs) and disseminated tumour cells (DTCs) might serve as prognostic markers to supplement routine staging and support determining individual therapeutic interventions. This review focuses on summarising the current knowledge about the detection of CTCs/DTCs with special emphasis on patients suffering from OSCC. The translational relevance of CTCs/DTCs and challenges for clinical application are highlighted.

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INTRODUCTION

Because of its biological heterogeneity and complex behaviour, head and neck cancer must be considered a conglomeration of several tumour types. The vast majority of patients are diagnosed with squamous cell carcinoma (SCC). With an estimated yearly incidence worldwide of half a million persons, nearly 50% of patients die due to tumour-related complications[1,2]. The disease ranks age dependently among the leading types of solid cancer and is responsible for more than 65000 annual deaths in Europe[3,4]. Early stage carcinomas are only observed in one third of the patients [Union for International Cancer Control stage (UICC) Ⅰ-Ⅱ] while two thirds present with advanced disease (UICC-Stage Ⅲ-Ⅳ). More than 50% of patients suffering from head neck squamous cell carcinoma (HNSCC) sustain local relapses while only up to 25% develop distant metastases. However, the prognosis of these patients is still poor[5-7].

Although the most important prognostic indicator for relapse is lymph node metastasis in the neck, the incidence of distant metastasis has increased dramatically[8,9]. Despite considerable advances in diagnosis[10] and therapeutic options[11], the 5-year survival rate has remained stable at approximately 50% during the past decades among all tumour stages[12,13]. This finding might also be due to an altered pattern of the clinical disease itself. While fewer patients currently present with a locoregional relapse, more patients develop a distant disease and recurrences due to occult cervical metastasis. These micrometastases are assumed to contribute to increased mortality and morbidity[14,15]. Thus, improved methods in staging oral squamous cell carcinoma (OSCC) are needed to detect early metastatic spread and any residual tumour, hence improving decisions about individual therapeutic interventions. The establishment of a resilient model of genetic instability, such as a loss of heterozygosity at distinct chromosomes and the consecutive progression from premalignant lesions to invasive tumours in head and neck cancer, led to a better understanding of the disease[2,16-19]. Due to the paradigm shift of considering solid cancers a systemic disease, the value and promise of circulating tumour cells (CTCs) in peripheral blood (PB) and disseminated tumour cells (DTCs) in bone marrow (BM) for cancer patients regarding diagnosis, prognosis, and response to therapy came into focus. While sampling BM from the easily accessible iliac crest is well established in diagnosing leukemic diseases, the full acceptance of this practice as an additional diagnostic tool for patients with solid tumours is still lacking. For carcinomas, including SCC, BM should operate as a so-called "homing organ" for CTCs becoming DTCs in the nomenclature. The clinical relevance of CTC/DTC detection in carcinoma patients has been part of extensive research, and encouraging results exist for an association between CTC detection and the prognosis in patients with other solid tumours, such as metastatic breast, prostate, and gastrointestinal cancers[20-28]. For HNSCC, a putative prognostic value was identified as well, revealing a positive correlation between CTCs/DTCs to relapse and metastasis[29-32]. In this context, it is important to note that the clinical behaviour and outcome of OSCC differ from those of the oropharynx and other HNSCC. Although there is no sharp distinction between OSCC and HNSCC in general, not all aspects and findings are transferrable. Even the main therapeutic options vary between the OSCC and other HNSCC. For OSCC, surgical removal is the primary treatment, whereas there is an ongoing debate about the preferred treatment options, such as primary radiation, (induction-) chemotherapy and supplemental targeted therapy, for laryngeal or nasopharyngeal SCC[33,34]. However, recently published data suggest that DTCs and CTCs in patients with OSCC are independent prognostic markers of disease-free survival[35]. Identifying clinical behaviour and understanding the inherent patterns of OSCC dissemination might result in meaningful contributions to the treatment of those patients. The application of new technologies that make the detection of CTCs/DTCs feasible might allow for more accurate staging and could help select patients and provide targeted therapies. To identify patients in need of systemic therapy in addition to local resection and irradiation, it is important to identify patients suffering from non-localised but "circulating" disease to expand the indication for systemic therapy. With new prognostic markers in prospect, the therapeutic options could substantially improve[36-38].

DETECTION AND CHARACTERISATION OF CTCS

Several techniques have been established to detect the rare CTCs/DTCs. To put the rarity of these cells in perspective, by applying currently available methods, it is estimated that there might be only one tumour cell among millions of other cells, even in metastatic disease. To increase the efficiency of detecting these cells, an initial enrichment step is needed. Basic enrichment approaches rely on either physical properties, such as size, density or dielectrophoretic mobility that might be characteristic at least for subgroups of CTCs/DTCs, or on the expression of specific surface molecules captured by magnetic bead-coated antibodies. Subsequently, one has to decide between cytometric/immunological approaches on one hand and molecular [mostly real-time reverse transcription-polymerase chain reaction (RT-PCR)-based] techniques on the other hand to detect the CTCs/DTCs among the simultaneously enriched non-tumoural cells[37].

A widely used method is gradient centrifugation using Ficoll-Hypaque, which utilises the density of different cell types. However, during enrichment, a substantial loss of cell-material is described[39]. The advantages of using immunocytochemical detection of enriched CTCs/DTCs include the possibility to characterise these cells by size and shape as well as by the nucleus-plasma relation. A set of monoclonal antibodies against various epithelial proteins is available, including various keratins of the cytoskeleton, surface adhesion molecules or growth fac-

tor receptors. The pan-keratin-antibody A45-B/B3 is still widely used for detecting DTCs *via* an alkaline phosphatase anti-alkaline phosphatase technique with New Fuchsin as a substrate[35,40]. Automated screening for potential CTCs/DTCs and visualisation can be performed with the ACIS™-system (Chromavision, San Juan Capistrano, CA, United States)[41,42].

Thus far, one of the most advanced methods for capturing and enumerating CTCs from PB is represented by the CellSearch™-system (Veridex, Raritan, NJ, United States), which provides automated enrichment and immunostaining of CTCs. It is the first food and drug administration-cleared device in CTC detection for solid cancers and has been approved for metastasised prostate, breast and colon cancers[23,43-46]. Gröbe *et al*[35] succeeded in detecting CTCs in a small subset of OSCC patients as well. The underlying principle uses immunomagnetic bead separation of EpCAM-positive tumour cells followed by immunofluorescent staining with anti-keratin antibodies and the exclusion of leukocytes using an anti-CD45-antibody. The CellSearch system benefits from selecting for epithelial features, such as the epithelial cell adhesion molecule (EpCAM) exposed at the surface of the vast majority of epithelial but not on normal blood cells.

A semi-automated process *via* fluorescence microscopy scans visualises the results. Subsequently, nucleated cells, with a diameter of at least 4 μm and characterised by keratin-positivity and CD45-negativity, are accepted and designated as epithelial cells as surrogates for tumour cells[20,44]. Other promising EpCAM-based tools were presented recently, including the microfluid-based "CTC"-chips consisting of arrays of anti-EpCAM antibody-coated microposts able to capture EpCAM-positive cells under controlled laminar flow of whole blood[47,48]. However, these techniques still lack tumour cell specificity and an application for head and neck tumours. Moreover, widespread application must be proven in large clinical trials.

Furthermore, other techniques were used for the detection of CTCs in patients with HNSCC. The Surfaced enhanced Raman Spectroscopy with epidermal growth factor receptor (EGFR) as a targeting ligand has been proven for the detection of putative CTCs in 19 patients by Wang *et al*[49]. The potential use of EGFR expression and activation in finding CTCs during the course of combined chemo- or bioradiotherapy regimens, accentuating the aspect of monitoring the therapeutic response in HNSCC, was described by Tinhofer *et al*[50] using flow cytometry[49-51]. Alternative approaches should be mentioned, however, these approaches still lack proof for their utilisation in OSCC. For example, the detection of CTCs from large blood volumes after leukapheresis is promising and paves the way for the analysis of higher numbers of CTCs either by fluorescence-activated cell sorting, immunocytochemistry or molecular approaches[52,53]. Moreover, real time monitoring of CTCs is desirable, such as during the course of therapeutic interventions. In this context, CTC detection *in vivo* is provided by the GILUPI cell collector device using a wire coated with anti-EpCAM antibodies ready to accumulate CTCs after insertion into a vein for 30 min. The detection of CTCs and the exclusion of leukocytes can be confirmed by immunostaining with anti-keratin and anti-CD45-antibodies, respectively[54]. To prove reliability and applicability for different tumour types and clinical relevance, several clinical trials are ongoing[37].

The detection of viable CTCs/DTCs after secretion of specific proteins during a 48 h short term culture is possible using epithelial immunospots (EPISPOT)[55]. For the EPISPOT approach, leukocytes must be depleted by negative selection of haematopoietic cells, such as using the common leukocyte antigen CD45.

To conclude, of the various introduced techniques, consideration should be given to molecular technologies. These technologies involve either DNA or complementary DNA (mRNA)-based PCR-amplification. These methods rely on gene expression patterns or the detection of known gene mutations, amplifications, other genomic aberrations or methylation patterns in tumour cells with the restriction that currently no universally applicable marker exists due to strong inter- and intra-tumoural heterogeneity. Nevertheless, using epithelium-specific targets, such as keratin 19 encoding transcripts, RT-PCR approaches are promising[56,57].

Wicha *et al*[58] have recently suggested, for breast and other carcinomas, that not all CTCs in these patients can be found with the approaches that are currently available and that not all detected CTCs are aggressive and have the potential to initiate metastases or recurrences, and these suggestions might also be true for OSCC. Therefore, strong efforts must be made to detect CTCs that have lost their epithelium-specific features and instead favour mesenchymal characteristics as the cells may be undergoing epithelial-mesenchymal transition (EMT). Investigations applying ultrasensitive imaging procedures, gene expression analyses and next generation sequencing approaches are ongoing and have already yielded interesting data[38,59-61].

A DISCUSSION OF THE RESULTS FROM STUDIES ON HEAD AND NECK CANCER

In comparison to other cancer types, the clinical impact of CTCs in OSCC and other HNSCC patients (Table 1) is still unclear. Therefore, investigating a correlation between these cells and clinico-pathological features is crucial. The research on the outcome of patients suffering from OSCC is impaired by comorbidities, such as chronic obstructive pulmonary disease, malnutrition, loss of function due to surgery and therapy, which can influence decisive parameters. Moreover, a sharp distinction between OSCC and HNSCC is hindered by similar but different causative pathogens, such as the contribution of human papillomaviruses (HPV) in the carcinogenesis of a subset of HNSCC, which is found less often in OSCC[62]. The incidence of the distinctly different disease

Table 1 Detection of circulating and disseminated tumour cells in oral squamous cell carcinoma and head neck squamous cell cancer

Source	Entity	*n*	Assay system	CTC-positive (%)	DTC-positive (%)	Clinical relevance	Number of analysed OSCC	Ref. yr of release
PB	OSCC	20	RT-PCR: CK19 mRNA	20.0		Detection of CK19-mRNA when tumour is incised	OSCC only	[85], 2000
PB	HNSCC	77	RT-PCR: CK19 mRNA	2.1		No significance for CTCs	Various sites *n* = 13 OSCC *n* = 23 oropharyngeal	[79], 2001
PB/ CVB BM	HNSCC	40	ICC: Keratins CK1-8, 10, 14-16 and 19 RT-PCR: E48 mRNA	10.0 (PCR) 20.0 (ICC)	20.0 (PCR) 32.0 (ICC)	E48 mRNA and DMFS/DFS preoperative in BM (P = 0.001/P = 0.002) and CVB (P < 0.001/P = 0.013) E48-mRNA and DMFS/ DFS intraoperative in CVB (P = 0.026/0.001) ICC and DMFS preoperative in BM (P = 0.0006) and CVB (P < 0.001) ICC and DFS preoperative in BM (P = 0.036) and CVB (P = 0.042)	OSCC only	[81], 2003
BM	HNSCC	162	RT-PCR: E48 mRNA		40.0	DTC on DFS (P = 0.1460) DMFS (P = 0.2912) N > or = 2 DMFS (P = 0.0210)	Various sites *n* = 13 OSCC *n* = 23 oropharyngeal	[80], 2004
BM	HNSCC	176	ICC: CK19		30.7	OS and N-involvement (P = 0.0001), OS and DTC (0.0066)	Various sites *n* = 37 OSCC *n* = 43 oropharyngeal	[29], 2004
PB	HNSCC	21	Immunomagnetic separation: EpCAM	33.3		Occurrence of CTCs more frequent in stage Ⅲ-Ⅳ than in stage Ⅰ-Ⅱ; connection with recurrence presumed; NS	Various sites *n* = 7 OSCC/ oropharyngeal	[83], 2007
PB	OSCC	25	RT-PCR: CK19 mRNA	16		Detection of CK19-mRNA when tumour is incised	OSCC only	[108], 2008
PB	HNSCC	48	Immunomagnetic separation: ICC: CK3-6H5	71		DFS and NO CTC (P = 0.01) clinical outcome and CTCs (P = 0.04)	Various sites *n* = 25 OSCC *n* = 10 oropharyngeal	[30], 2010
PB	HNSCC	42	Flow cytometry: EpCAM, CD45 RT-PCR: EGFR mRNA	43		CTC and N in flow cytometry (P = 0.014), CTC and N in RT-PCR	Various sites *n* = 9 OSCC *n* = 17 oropharyngeal	[31], 2011
PB	HNSCC	15	CellSearch™	40		CTC and M (P = 0.04)	Various sites *n* = 4 OSCC *n* =7 oropharyngeal	[84], 2012
PB	HNSCC	31	Flow cytometry: EpCAM; EGFR, CD45	29 pEGFR in 55% of CTC^+-cases		Increasing number of CTC-positive cases at end of treatment	Various sites *n* =5 OSCC *n* =18 oropharyngeal	[50], 2012
PB	Under RCT HNSCC	33	Flow cytometry: EpCAM CD45 keratins 7, 8 EGFR	33 pEGFR in 36.4% of CTC^+-cases		None given for comparing two methods	Various sites *n* = 0 OSCC (not specified) *n* = 13 oropharyngeal	[51], 2012
PB	HNSCC	73	CellSearch™	15.1		Decreasing CTC counts and clinical response (P = 0.017)	Various sites *n* = 3 OSCC *n* = 39 oropharyngeal	[82], 2012
BM	HNSCC	105	ICC: keratins 8, 18 and 19		14.5	No significance for DTCs (76/105 tested)	Various sites *n* = 72 OSCC *n* = 8 oropharyngeal	[68], 2012
PB BM	OSCC	110	CellSearch™ ICC: keratins 8, 18 and 19	12.5 (Cell search)	20.0 (ICC)	CTCs and T (P = 0.04), N and DTCs (P = 0.02), M and CTCs (P = 0.004), M and DTCs (P = 0.005), DTCs and RFS (P < 0.001)	OSCC only	[35], 2014

BM: Bone marrow aspirates; CD: Cluster of differentiation; CK: Cytokeratin; CTC: Circulating tumour cell; CVB: Central venous blood; DFS: Disease-free survival; DMFS: Distant metastasis free survival; EGFR: Epidermal growth factor receptor; pEGFR: Phospho-EGFR; HNSCC: Head neck squamous cell cancer; EpCAM: Epithelial cell adhesion molecule; M: Metastatic; NS: Nodal status; OS: Overall survival; OSCC: Oral squamous cell cancer; PB: Peripheral blood; RT-PCR: Reverse transcriptase-polymerase chain reaction; RFS: Relapse-free survival; T: Tumour stage; ICC: Immunocytochemistry; RCT: Randomized control trial; DTC: Disseminated tumour cells.

of HPV-related head and neck cancers from other head and neck carcinomas is increasing[63]. This finding will influence further studies regarding etiology and outcome and therefore must be included when evaluating these studies, especially those of patients undergoing different therapy-regimens.

With reference to the concept of field cancerisation (FC) introduced by Slaughter *et al*[64] in the 1950's, related problems in OSCC should also be considered because locoregional failure can lead to distant metastasis in HNSCC[65]. FC is thought to be undetectable by routine procedures thereby potentially causing a second primary tumour[66]. Furthermore, the entire mucous membrane of the respiratory and upper digestive tract is at risk for neoplasia[67]. Graveland *et al*[68] investigated the potential association of minimal residual cancer in deep surgical margins and DTCs in BM aspirates with clinico-histopathological parameters and outcomes and concluded that the presence of vasoinvasive and

infiltrative growth in HNSCC is a significant risk factor for locoregional recurrence and disease-free survival. Furthermore, they concluded that, at present, there seemed to be no role for the molecular analysis of deep surgical margins and BM aspirates in predicting outcomes with the methods used for 105 HNSCC patients enrolled in this study. To improve histopathological staging in previously tumour cell-negative margins, the overexpression of p53 frequently found in primary tumours was revealed to be indicative of an increased risk of local recurrence[69,70]. Clinically relevant tumour cell spread is rarely found beyond the level of histopathological detection. Thus, a molecular assessment of minimal residual disease is needed to fill the gap between histopathological findings and outcomes in HNSCC, including OSCC[71]. Several serum tumour markers have been evaluated with regard to clinical value in HNSCC and OSCC, but most of them demonstrated poor sensitivity[72,73]. This article focuses on the potential haematogenous spread of tumour cells while OSCC most often spreads in lymphatic vessels[74-76], potentially decreasing the relevance regarding OSCC/HNSCC. However, there are increasing signs that CTCs are clinically relevant in the pathogenesis and recurrence of head and neck tumours. The total number of suspicious cells most likely depends on the method applied. OSCC is characterised by specific expression patterns for different keratins, capable of being detected in CTCs/DTCs[77], leading to technical limitations that must be considered. The detection methods have been optimised for CTCs or DTCs derived from adenocarcinomas such as breast, prostate, colorectal or hepatocellular carcinomas, as reported by Schulze *et al*[78] in the latter case. Therefore, an adaption to the unique biology of OSCC may be required.

Nevertheless, one of the first reports of prognostic value in a homogenous group of OSCC has been recently published by Gröbe *et al*[35], which describes a strong and independent prognostic impact of CTCs and DTCs for disease-free survival, which outperformed current prognosticators. This finding might provide evidence that patients who are positive for tumour cells in the BM or PB may suffer from a more aggressive disease. Thus, CTCs/DTCs detected in OSCC patients might serve as prognostic markers, predicting relapse at various disease stages, supplementing the current routine staging procedures. In this study, 110 patients were enrolled. Anti-keratin immunostaining after Ficoll density gradient centrifugation to detect DTCs and applying the CellSearch™-System to analyse PB represent techniques with the current highest degree of international standardisation[42,44]. Nineteen patients (21%) exhibited DTCs in the BM, while 11 patients (13.6%) were positive for CTCs in the PB. According to other literature data, CTCs/DTCs are traceable in the PB of HNSCC patients[29,31]. Wollenberg *et al*[29], for example, detected DTCs from BM aspirates, depending on tumour stage.

Gröbe *et al*[35] found no significant correlation between the detection of CTCs and the presence of DTCs (P = 0.68), suggesting that both analyses might provide supplementary results. Distant haematogenous metastases were less frequently observed compared to locoregional relapses. Consistent with other studies examining more heterogeneous groups of HNSCC patients[29,79,80], the subsequent development of recurrent disease was observed more frequently when patients were positive for CTCs/DTCs[35,81]. However, there are several incongruous and moderately significant results demonstrating that the higher the disease stage, the higher the yield of detected CTCs. Although statistically not significant, a trend regarding this conclusion was shown by Buglione *et al*[82] last year, comparing T1 to higher tumour stages in HNSCC, but in the OSCC subgroup (13/152 patients), no CTCs were found. Previously, Guney *et al*[83] found similar results for HNSCC but in a smaller cohort of patients. A correlation to tumour stage was found, though not significant for DTCs, and the total number of CTCs was higher in T3-4 tumours compared to T1-2 tumours[29]. These findings suggest that the size of the primary tumour is not always prognostically relevant. This hypothesis is strengthened by significantly higher incidences of CTC positivity in patients with locally advanced disease when considering the N-stage, holding true even in a multivariate analysis[31]. Moreover, CTC detection was significantly associated with the presence of lung nodules[84]. The tumour mass limits local resection by potentially decreasing safety margins because of neighbouring structures, such as the sinus at the skull base, which reaffirms the problem of an assumed CF. Nevertheless, a longer disease-free survival of patients without detectable CTCs compared to CTC-positive patients was stated by Jatana *et al*[32]. Unfortunately, most of the studies cited were performed on small patient cohorts for other groups of HNSCC[83,84]. In a distinctly larger group of 176 HNSCC patients, the presence of DTCs in BM showed prognostic relevance for overall survival. In half of the DTC-positive patients, the disease recurred, whereas in the total cohort, recurrences were only observed in 27% of the patients[29].

The importance of CTC detection in the context of therapy is still controversially discussed. For OSCC patients, there are studies investigating the dissemination of tumour cells after surgery and tumour cell spread due to surgical procedures[32,85], but among these studies, only a few have investigated the impact of metastases and survival regarding this issue. These facts are crucial in OSCC as surgery is the current gold standard and primary treatment option.

Before, during and following radiotherapy the detection of keratin-19-positive cells coincided with local failure, distant metastasis and moreover, the diagnosis of anaemia[79]. Regarding the therapeutic response in HNSCC with decreased levels of previously detected CTCs, a positive effect on outcome was presumed by Buglione *et al*[82] because partial or complete response could be stated along with these findings.

A fact that might impair the analysis of detected CTCs/DTCs is the subjective evaluation by even experienced independent readers of common immunocytochemical methods, such as the use of the anti-keratin

antibody A45-B/B3. However, false-positive results are seldom found[86]. The criteria to evaluate the morphology of potential CTCs and immunostaining reactions are crucial in defining comparable results[39,42]. This knowledge is even important to evaluate results obtained for blood samples analysed with the semi-automated, highly standardised CellSearch system. To overcome these problems, different international ring studies have begun to reduce interobserver variability of the results[44,87]. The successful CellSearch system has proven its usefulness in OSCC/HNSCC, although not in all advanced stage patients. However, even in metastasised, advanced disease, CTCs were not detectable in all cases[35]. Nichols *et al*[84] detected almost 100% of spiked HNSCC cell line cells (FaDu) using the CellSearch system, however only 6 out of 15 HNSCC patients tested CTC-positive using this approach. The results are in accordance with those of other studies but suggest that, in patients, either the incidence or number of potentially detectable CTCs is very low or that not all present CTCs are detectable with the currently applied approaches. Furthermore, the system is ruling out non-EpCAM and/or non-keratin expressing CTCs. This escape from detection has been previously demonstrated for breast cancer where, in aggressive tumours, EpCAM expression might be down-regulated, and this finding has also been assumed for aggressive forms of solid cancers other than OSCC[88].

Hence, serious consideration should be given to EMT, which is associated with a loss of epithelial features, enhancing the migratory capability due to altered plasticity[88,89]. Stem cell-like cells seem to be most notably affected, as dynamic changes in these cells were identified by Mani *et al*[90] and Yu *et al*[91]. Thus, increasingly false-negative results might correlate to EMT[92]. The detection of EpCAM-negative cells might be possible by using other surface markers, such as N-cadherin, EGFR or cancer stem cell markers, such as CD44[89,93,94]. In colorectal carcinomas, plastin3 was identified as a potential target for cells undergoing EMT because its expression is not altered during this process[95]. Superiority over EpCAM-based methods must be proven, and expression of plastin3 in cancers other than colorectal cancer must be analysed. EGFR, frequently overexpressed in HNSCC, is a meaningful target for CTC enrichment[50,96]. Due to the biological heterogeneity of CTCs/DTCs, the most aggressive subsets initiating metastasis must still be categorised[97,98]. In OSCC, Nagata *et al*[99] performed molecular analyses, identifying integrins as potential biomarkers for the risk of locoregional and haematogenous dissemination. The role of the expression rates of integrin alpha-3 for locoregional and ITGB4 for haematogenous dissemination and, therefore, cancer cell motility and anchorage-independent survival, which are vital for OSCC recurrence and metastasis, has previously been shown. Furthermore, the importance of integrin beta1 in HNSCC and stimulated vascular endothelial growth factor secretion by alphaB-crystallin in HNSCC was specified[100,101].

In addition to these considerations, there might be a spread of tumour-derived molecules on a subcellular level, such as micro-RNAs. Their role in the formation of metastases derived from OSCC is currently unclear[102,103].

According to current hypotheses, most DTCs remain in a dormant state and probably never initiate a relapse or metastasis[56,104], whereas others return to a proliferating state by still unknown molecular alterations or changes in environmental conditions[105,106]. Interestingly, also a re-circulation from the dormant site back to the primary location of the cancer has been reported in an mouse breast cancer model[107], but because of the high rate of locoregional failure, the concept does not seem apparent for OSCC in the reviewers opinion due to the concept of FC[67]. Therefore, more obvious challenges must be faced when considering of this phenomenon. The spread of tumour cells during surgery remains unclear but has been proven for fine needle aspiration or incision biopsy in OSCC[85,108]. A total of 71% of 48 patients harboured CTCs detected by Jatana *et al*[30] in HNSCC. Here, samples were taken at the time of surgery. Circulating epithelial cells also can be found in non-malignant diseases, such as inflammatory diseases of the colon, with the current assays[109].

Nevertheless, the appealing potential of detecting CTCs/DTCs as prognostic markers in OSCC from non-invasively accessible blood samples must be emphasised. When occult tumour cells are ubiquitously spread, PB may be a way to detect these cells, facilitating a "liquid biopsy"[37]. Out of a set of several markers, a decision must be made, and the occurrence of CTCs is a very rare event. Researchers are faced with the problem that often processed samples cannot be used a second time for other methods, and the samples display only a cross section of the entire circulating blood. A reliable, morphological identification, for example, with fixation of the cells and nuclear staining might prevent subsequent molecular characterisation. These analyses provide just a snapshot of tumour cell dissemination in contrast to BM, which may represent a reservoir where DTCs can be collected over a longer period of time[104]. Many CTCs are assumed to undergo apoptosis quickly[110], as the lifespan of CTCs in the circulation is estimated to be only between one and two hours[111]. Alternative approaches, such as the "CTC-Chip", punctuate an improvement in CTC yield and purity but only give a possible outlook due to the small number of examined cohorts and have not been tested for OSCC yet[47,112].

To identify potential targets for individualised therapies and to use repeated CTC assessments in individual patients for treatment surveillance is of the utmost importance[37]. A potential marker in breast cancer is the human epidermal growth factor receptor 2 (HER2), the expression of which can also be monitored on CTCs, thus giving patients with HER2-negative primary tumours but HER2-positive CTCs a chance to be treated by a HER2-targeted therapy[113,114]. Currently, there is no biomarker available for OSCC yet. BM might be an interesting target organ for therapeutic interventions by considering DTCs as therapeutic targets. The data described here point to the potential future utility of drugs targeting DTCs in the

BM of OSCC patients. The present high expenses will decrease when evidence is found that these methods are suitable for OSCC and can be included in standard protocols for tumour staging.

CONCLUSION

A set of promising techniques for the detection of CTCs/DTCs has been developed in the past decade, proving their usefulness in solid cancers with predominantly haematogenous dissemination. Less invasive diagnostic tools might be appropriate to refine the current staging methods. The non-invasiveness of collecting samples from PB and obtaining BM specimens might provide additional treatment options in patients suffering from OSCC. For OSCC, the current data show that CTCs/DTCs might have a clinical impact on the prognosis and survival, even outperforming current prognosticators. Circulating tumor cells and DTCs must be considered independent markers in OSCC as a subgroup of head and neck tumours, but the ongoing debate remains too controversial to be considered evidence. The findings presented in this review are not convincing for oral cancer in all aspects yet. Nevertheless, the potential is striking, guiding therapy towards individual-centred medicine with the possibility of early intervention in this severe disease. Despite encouraging results, further investigation of the clinical relevance of CTCs and DTCs is needed as validation in large multicentre trials with synchronised measuring methods and defined outcomes and time points is lacking. In particular, the risk of an early locoregional relapse is difficult to assess because of different problems in the field of HNSCC and OSCC. It is important to continue investigations in homogenous cohorts to decrease the still unaltered morbidity in patients suffering from OSCC. Due to being one of the most active areas of translational cancer research, the technologies presented herein offer a number of compelling advantages. The enumeration of CTCs and DTCs as biomarkers to predict tumour relapse may become feasible in the near future, but researchers in this extraordinarily dynamic field are still facing technical and clinical hurdles. In addition to further improvements, there are challenges beyond the mere detection of CTCs/DTCs. In particular, the detection of carcinoma cells undergoing EMT, a down-regulation of epithelial features when entering vessels, the transient circulation of cells, the mechanisms behind leaving the "homing organs" and the states of dormancy need to be investigated. Whether EpCAM- or keratin-positive cells detected by the described techniques are involved in initiating metastasis is also controversially discussed. To address this question further, molecular analysis of CTCs/DTCs is needed.

A profound understanding of OSCC as a systemic disease supported by molecular investigation of CTCs/DTCs gives new perspectives on the disease and provides the chance to identify potential targets for individualised therapies. Repeated CTC assessments might offer treatment surveillance in individual patients prospectively and might predict local and systemic relapse with a higher sensitivity at various disease stages compared to routine staging procedures in OSCC.

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