Prognostic Relevance of Circulating Tumor Cells in Blood and Disseminated Tumor Cells in Bone Marrow of Patients with Squamous Cell Carcinoma of the Oral Cavity

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Abstract

**Purpose:** Current staging methods for squamous cell carcinomas (SCC) of the oral cavity (OSCC) need to be improved to predict the risk of individual patients. Because hematogenous tumor cell dissemination is a key event in tumor progression, we assessed the prognostic significance of disseminated tumor cells (DTC) in bone marrow and circulating tumor cells (CTC) in peripheral blood from patients with OSCC.

**Experimental Design:** From 110 patients with OSCC, tumors were surgically resected (R0) without neoadjuvant therapy. The CellSearch system was used to enumerate CTCs. Bone marrow was aspirated from the iliac crest, and mononuclear cells (MNC) were enriched by Ficoll density gradient centrifugation. To detect DTCs, MNCs were immunostained with the pan-keratin antibody A45-B/B3. Results were correlated with clinicopathologic parameters and clinical outcome such as recurrence and death during follow-up time (mean 916 days).

**Results:** Ten of 80 patients (12.5%) harbored CTCs in peripheral blood, whereas in 18 of 90 patients (20.0%) DTCs in bone marrow could be detected. Surprisingly, in only 2 patients (1.8%) CTCs and DTCs were detected simultaneously. Significant correlations could be found for CTCs and tumor size ($P = 0.04$), nodal status and DTCs ($P = 0.02$), and distant metastasis with CTCs ($P = 0.004$) and DTCs ($P = 0.005$). Univariate and multivariate analyses revealed that CTCs and DTCs were significant and independent predictors of recurrence-free survival ($P < 0.001$).

**Conclusions:** Both DTCs and CTCs are independent prognostic markers in patients with OSCC, predicting relapse with higher sensitivity at various disease stages than routine staging procedures. Bone marrow might be an interesting target organ for future therapeutic interventions. *Clin Cancer Res;* 20(2); 425–33. ©2013 AACR.

Introduction

Oral squamous cell carcinoma (OSCC) is the fourth leading cancer behind lung, breast, and colon carcinoma and the eighth leading cause of cancer-related death worldwide (1, 2). It is the most frequent histologic type representing approximately 95% of head and neck cancer. The estimated incidence worldwide is approximately 550,000 cases and almost 50% of the patients die of the disease (3).

Only about one third of the patients present with early-stage disease (Union Internationale Contra Cancrum, UICC, stage I–II), whereas two thirds show already advanced disease (UICC stage III–IV) with poor outcome. The prognosis of patients suffering from head and neck SCC (HNSCC) remains poor and the risk to develop local relapse is higher than 50%. Furthermore, the development of distant metastasis occurs in 15% to 25% of the patients (4, 5). The most important prognostic indicator for relapse of OSCC is the presence of metastatic spread to lymph nodes in the neck. In this case, the incidence of distant metastases can be as high as 50% (6–9).

Despite improvements in diagnosis and therapeutic concepts the 5-year overall survival rate has not improved significantly over the last 25 years and remains stable around 56% (10, 11). One reason is a change in the pattern of clinical disease with fewer cases suffering from locoregional relapse, but more patients develop distant metastases, which are an increasing cause of morbidity and mortality (12).
Translational Relevance

Current staging methods for oral squamous cell carcinomas (OSCC) need to be improved to detect early metastatic spread and clarify the individual need of therapeutic interventions. This study indicates that circulating tumor cells/disseminated tumor cells (CTC/DTC) detected in patients with OSCC serve as prognostic markers, predicting relapse at various disease stages supplementary to routine staging procedures. Interestingly, there was little overlap between tumor cell detection in the peripheral blood and bone marrow, indicating that both compartments offer complementary diagnostic information on tumor cell spread in patients with OSCC. These findings point to the potential future utility of drugs targeting DTCs in bone marrow and CTCs in blood of patients with OSCC.

Over the past 20 years, the presence of disseminated tumor cells (DTC) in bone marrow has been shown to be a putative prognostic indicator for patients with various kinds of solid epithelial tumors, including HNSCC, and thus have been part of extensive scientific research (13). Previous studies on bone marrow samples from patients out of the heterogeneous group of HNSCC—including oral, pharyngeal, laryngeal, and skin squamous cell carcinomas (SCC)—revealed a positive correlation of DTC detection to relapse and metastases (14).

However, because repeated and sequential bone marrow analyses are only possible in selected patients during follow-up observations, analysis of peripheral blood has become a promising alternative to investigate tumor cell dissemination (15). Especially for patients with metastatic carcinomas, the presence of circulating tumor cells (CTC) in peripheral blood is associated with a worse prognosis (16–18). In only a limited number of mainly breast cancer studies the presence of DTCs in the bone marrow and peripheral venous blood in the same patients was analyzed and all of these studies displayed discrepancies in positivity rates up to approximately 85% overlap (19), suggesting that the detection of CTCs provides complementary information to bone marrow analysis.

As recent studies suggested, CTCs might be an interesting independent prognostic marker in HNSCC as well (20, 21). Interestingly, the clinical behavior and outcome of tumors in the oral cavity is distinct from those of the oropharynx and other HNSCC. Briefly, surgical resection is the primary treatment for OSCC, whereas the preferred treatment with primary radiotherapy, (induction-) chemotherapy, and/or targeted therapy for laryngeal or nasopharyngeal SCCs depending on stage is under discussion (22, 23). A clear understanding of the anatomy and knowledge of clinical behavior and spread patterns of OSCC are essential to make a meaningful contribution to the treatment of these patients and might allow more accurate staging and selection of patients for whom new target therapies are an option. Thus, systemic spread of tumor cells implies systemic therapy and not only local radiotherapy. Therefore, it is indispensable to detect patients suffering from systemic tumor spread to expand the indication for systemic therapy.

The aim of the present study was to assess both, DTCs in bone marrow and CTCs in peripheral blood of patients suffering from OSCC, and to correlate these findings with the size of the tumor (T-stage), locoregional spread (N-stage), distant metastases (M-stage), UICC-stage, and the prognosis (recurrence and survival) of these patients.

Patients and Methods

Patients

The present study was approved by the Medical Ethical Committee (Hamburg, Germany) and complies with the principles laid down in the Declaration of Helsinki. All subjects gave informed consent to the work. Of note, 110 patients (36 female, 74 male; ages 40–83 years; arithmetic mean 54.7 years) were investigated with histologically diagnosed SCC of the oral cavity (Table 1). Tumor size, locoregional spread, and distant metastases were determined according to the tumor-node-metastasis classification. Therefore, staging examinations included computed tomography (CT) scan of the head and neck and dependent on stage either X-ray of the lung and ultrasound of the abdomen for UICC 1+2 patients or CT scan of the thorax and abdomen in case of UICC 3+4. Only patients without neoadjuvant therapy were included in the study. All patients underwent primary surgical treatment for removal of the tumor with tumor-free resection margins (R0 resection) between 2006 and 2011 and depending on stage with subsequent radiotherapy and/or chemotherapy. The human papilloma virus (HPV) status could be examined in 54 patients and revealed positive results in 4 cases (7.4%, HPV-16) without any statistical association to clinicopathologic parameters, overall or disease-specific survival and CTC/DTC findings.

Bone marrow aspirates from the iliac crest and blood samples were taken before surgery after written informed consent. In total, we received 90 samples of bone marrow aspirates and 80 blood samples. In 17 cases we had the consent of the patient of solely taking the blood sample and in 30 patients we were only allowed to aspirate bone marrow and in 61 cases we had both. During follow-up, patients initially were investigated every 3 months and after 30 months every 6 months up to 5 years.

Detection of CTCs in peripheral blood

Capturing and enumeration of CTCs from peripheral blood was performed with the CellSearch system (Veridex; refs. 16, 24). Samples (7.5 mL) were collected in CellSave tubes (Veridex) and stored or shipped at room temperature. The CellSearch system consists of an automated instrument for capturing and immunostaining of CTCs (AutoPrep; Veridex), and a semiautomated fluorescence microscope for scanning and visualizing of the results (CellSpotter Analyzer; Veridex). Blood samples were...
processed with the CellSearch Epithelial cell Kit (Veridex). DAPI (4',6-diamidino-2-phenylindole) and cytokeratin-positive, but CD45-negative cells with a diameter of at least 4 μm were designated epithelial cells as surrogate for tumor cells (Fig. 1A; refs. 16, 24).

Detection of DTCs in bone marrow
Bone marrow aspiration from the upper iliac crest during primary surgery, enrichment of mononuclear cells (MNC) by Ficoll density gradient centrifugation, preparation of cytopsins, and immunostaining were performed as described elsewhere (25–27). Nucleated cells that expressed a common epitope of various keratins, including keratins 8, 18, and 19, as demonstrated by positivity for the pan anti-keratin antibody A45-B/B3 (mouse IgG1; AS Diagnostics; Fig. 1B), were counted as DTCs. After incubation of two million aceton–fixed MNCs from each patient with blocking serum (Biotest; diluted 1:10 in PBS) for 20 minutes, incubation with A45-B/B3 (2 μg/mL in PBS solution with 10% AB serum) was performed. A polyclonal rabbit anti-mouse antibody (DAKO) and a mouse alkaline phosphatase–anti-alkaline phosphatase antibody (DAKO) served as secondary and tertiary antibodies, respectively. Visualizing of A45-B/B3-positive cells was facilitated by a red color reaction involving New Fuchsin, naphtol-AS biphosphate and levamisole. Finally, nuclei were counterstained with hematoxylin (Mayer’s hemalaun solution; Merck) and subsequently the slides were mounted in Kaiser’s glyceral–gelatin (Chroma Gesellschaft Gmbh). Stained slides were automatically screened for A45-B/B3–positive cells by the ACIS system (Chromavision). The criteria for categorization as DTCs were described elsewhere (25, 27, 28). These cells have to be keratin-positive with an enlarged nucleus or high nuclear to cytoplasmic ratio. Also defect suspicious tumor cells were counted. No immunostained cells with such morphology should be detected in the corresponding negative controls (MOPC-21; Sigma Chemical) monoclonal antibody lacking any known reactivity for human cells at the same concentration as the A45-B/B3 antibody was used (25). All A45-B/B3–positive cells were evaluated by 2 experienced readers, independently and discrepant results were discussed until consent was reached.

Statistical analysis
The acquired results were statistically analyzed using the computer program “R,” version 2.15.2. P values shown were estimated by using the χ² test. Descriptive statistics were used for baseline characteristics of the patients and potential associations between the CTCs/DTCs and clinicopathologic parameters were evaluated. Survival curves for disease-free and overall survivals of the patients were plotted using the Kaplan–Meier method and analyzed using the log-rank test. A Cox model was calculated and the HRs were presented as 95% confidence interval (CI). The overall survival was computed as the time period from the date of surgery to either the date of death or last follow-up, whichever occurred first. The disease-free survival was defined as the time period from the date of surgery to the date of recurrence or last follow-up, whichever occurred first. Patients alive or dead without recurrence at the last follow-up dates were censored. Cox regression hazard model was used for multivariate analysis to assess the independent influence of CTC/DTC and other covariates on tumor recurrence and overall survival. Results are presented as HR with 95% CI. Significant statements refer to P values of two-tailed tests that were P < 0.05.

Results
Correlation of the presence of CTCs to clinicopathologic parameters, relapse, and survival
In total, 110 patients were enrolled in the present study. Patients with MX stadium did not agree to the whole set of staging examinations, so the M status was initially unknown. Patients with an initial M1 status suffered from metastases in either the lung or liver. During the follow-up period, 91 patients (82.3%) emerged no relapse of the disease, whereas the remaining 19 patients (17.7%) developed a locoregional relapse. Patients were visited every third month after therapy for 30 months and every sixth month for another 30 months to reach a follow-up period of 5 years in total. Mean follow-up time of our patient samples was 30.1 months with a minimum of 0.2 months and a maximum of 178.7 months (median 19.7 months).
We analyzed peripheral blood samples from 80 patients. CTCs could be detected in 10 of 80 patients (12.5%; Fig. 1A), and cell counts ranged between 1 and 14 CTCs per 7.5 mL (mean: 3.2 CTCs/7.5 mL). Results of DTC and CTC detection in relation to clinicopathologic parameters are summarized in Table 2. Advanced tumor sizes (pT, $P = 0.04$) and distant metastasis (M, $P = 0.004$) significantly correlated with the detection of CTCs (Table 2, $P$ values as indicated).

No statistically significant correlation ($\chi^2$ test) could be found between the detection of CTCs and nodal status (pN, $P = 0.97$) and UICC stadium ($P = 0.71$ as indicated in Table 2).

Figure 2A demonstrates the association of CTC detection and the occurrence of locoregional relapses using Kaplan–Meier analysis. The cohort analyzed included 65 acquired patients. All patients who died during follow-up without a recurrence were excluded from the analysis, resulting in the definition of recurrence-free survival. Within the group of 10 patients with CTCs, 5 patients (50.0%) developed a locoregional relapse, whereas such relapse was only observed in 6 (8.6%) of 70 patients without CTCs. Detection of CTCs had a significant impact on reduced relapse-free survival both in the univariate and multivariate analysis ($P < 0.001$), including the presence of CTCs, DTCs, T-stage, N-stage, and M-stage. In the subgroup of 19 patients (17.3%) with a locoregional relapse during the follow-up period, 7 patients (36.8%) were CTC-positive, whereas only 3 patients (3.3%) were CTC-positive in patients without relapse ($n = 91; P = 0.0003$).

No statistically significant correlation ($\chi^2$ test) could be found between the detection of CTCs and overall survival of the patients ($P = 0.69$; data not shown), neither had the postoperative treatment modality (none, radiotherapy, concomitant radiotherapy/chemotherapy) any significant effect on locoregional relapse during follow-up (data not shown). Also, we found no correlation between the number of CTCs and the time to relapse or death ($P = 0.29$ for relapse and $P = 0.37$ for death), i.e., earlier relapse or death was not observed with increasing CTC numbers.

Next, we performed a multivariate analysis according to the Cox proportional hazard model using CTCs, DTCs, tumor size, lymph node status, and distant metastases because these parameters were statistically significant in our univariate analysis for relapse-free survival.

The presence of CTCs was found to be the strongest independent predictor of locoregional tumor relapse, even if patients with distant metastasis (M1) at diagnosis were excluded from the analysis ($P = 0.027$). The OR for CTC-positive patients was 56.06 with $P = 0.01$.

Correlation of the detection of DTCs to clinicopathologic parameters, relapse, and survival

Eighteen of 90 patients (20%) displayed DTCs in their bone marrow (Fig. 1B). DTC numbers varied from 1 to $27/2 \times 10^5$ MNC (3.2 DTCs/2 $\times 10^5$ MNC in mean).

Interestingly, the detection of DTCs correlated both significantly with a locoregional progressive disease (cervical lymph node involvement) and the presence of distant metastases (pN, $P = 0.02$ and M, $P = 0.005$, respectively; Table 3).
Prognostic Relevance of CTCs and DTCs in patients suffering from oral squamous cell carcinomas

Lack of correlation between detection of CTCs and DTCs

In 61 patients, we were able to analyze both blood and bone marrow samples. Surprisingly, we did not find a correlation between the presence of CTCs and DTCs (P = 0.68). Within this cohort, only 2 patients presented with positive results for both CTCs and DTCs. Interestingly, both patients developed a locoregional relapse during follow-up after 2 months and 2 years, respectively. One patient suffered from a progressive disease with a pT14 local tumor and regional tumor spread (pN2) without any signs of distant metastases. The other patient presented with a pT2 tumor without locoregional or distant tumor spread (pN0 and M0) after staging examinations. On the other hand, only 6 patients (6.6%) with locoregional tumor relapse were negative for either CTCs or DTCs.

We subsequently analyzed the prognostic value of combined CTC/DTC detection and had a statistically significant association between the combination of CTCs and DTCs and recurrence-free (P < 0.001) but not overall survival (P = 0.99; data not shown).

Discussion

The aim of the present study was to examine DTCs and CTCs in a rather homogenous group of patients with OSCC. Our key finding is the strong and independent prognostic impact of CTC and DTC detection for disease-free survival, which outperformed current prognosticators.

Data about CTC detection with the CellSearch system in patients with head and neck cancer have already been published; however, patient cohorts were heterogeneous with SCCs of different subanatomical regions, including hypo- and oropharyngeal cancer (29, 30). Interestingly, absence or disappearance of CTCs during therapy was shown to be associated with partial or complete response to therapy (29). Furthermore, similar to our findings, CTCs were more frequently detected in patients with advanced-staged tumors (T4 vs. T1–3; 29) and positivity rates were comparable between these and our studies.

In our study, 18 patients (20%) exhibited DTCs in the bone marrow, whereas 10 patients (12.5%) were detected positive for CTCs in the peripheral blood, which is in accordance to literature data (14, 21). Expecting ubiquitous spread of occult tumor cells, peripheral blood should be an excellent compartment to detect these cells (“liquid biopsy”). However, similar to patients with breast cancer we detected a higher frequency of bone marrow–positive aspirates than peripheral blood–positive samples (31). The half-life of CTCs in the circulation with 1 to 2 hours seems to be short (32) and hence many CTCs rapidly undergo apoptosis. On the other hand, the presence of DTCs was also found to be the strongest independent predictor of locoregional tumor relapse and addition of metastatic relapse. The OR for DTC-positive patients was 49.13 with P = 0.004. Similar to the presence of CTCs, DTCs (OR, 0.64; P = 0.76) were not significantly correlated with the overall survival of the patients.

Of the 19 patients with a locoregional relapse during follow-up, 9 (47.4%) were detected DTC-positive. In contrast, in only 10 (11%) of 91 patients without any signs of relapse DTCs were found. On the other hand, 9 patients (9.5%) developed a pulmonary distant metastatic relapse, of whom 5 patients (55.6%) were DTC-positive and 4 patients (44.4%) had no DTCs. The results of the Kaplan–Meier analysis were statistically significant for the locoregional relapse (P < 0.01) as well as the distant metastatic relapse (P < 0.001, as indicated in Fig. 2B and C) and in addition the result remains statistically significant after excluding all patients (n = 4) from the analysis (P < 0.001). Again, all patients who died during follow-up without a recurrence were excluded as defined for relapse-free survival.

In univariate analysis (χ² test), we did not observe statistical correlations between detection and overall survival (P = 0.34), size of the tumor (pT, P = 0.16), and UICC-stadium (P = 0.18; Table 3). Also, the postoperative adjuvant treatment modalities revealed only a statistically tendency but no significant results on locoregional relapse (data not shown). Furthermore, the number of DTCs had no impact on the time to relapse or death (P = 0.18 and P = 0.19, respectively).

Performing the multivariate analysis according to the Cox proportional hazard model (Fig. 3), the presence of DTCs was also found to be the strongest independent predictor of locoregional tumor relapse and additionally of metastatic relapse. The OR for DTC-positive patients was 49.13 with P = 0.004. Similar to the presence of CTCs, DTCs (OR, 0.64; P = 0.76) were not significantly correlated with the overall survival of the patients.
The association between the number of CTCs/DTCs and the time to relapse or death, possibly due to the small sample size of 10 and 18 patients, respectively, detected positive for CTCs or DTCs. These findings are consistent even if patients with metastatic disease at the time of diagnosis were excluded from the analysis and compatible to other studies (14, 34, 35) examining, however, a more heterogeneous group of patients with HNSCC. Our present study is, therefore, the first report on the prognostic value of CTCs and DTCs in a homogeneous group of patients with OSCC, which is of high clinical relevance. Thus, the detection of DTCs/CTCs might serve as a prognostic tool providing a more accurate individual risk profile of the single patient with higher sensitivity at various disease stages than routine staging procedures. Overall survival of the patients seems to be independent from CTC/DTC detection possibly due to a
rather short follow-up time in some cases or death caused by comorbidities. Finally, the detection of DTCs and CTCs was significantly associated with the presence of distant metastases. Because, in addition, postoperative treatment modalities such as radiotherapy or concomitant radiotherapy/chemotherapy revealed no significant impact on locoregional recurrence, this indicates that patients positive for tumor cells in the bone marrow or peripheral blood may suffer from a more aggressive disease. However, both detection methods have been optimized for the detection of CTCs or DTCs derived from adenocarcinomas and, therefore, may require adaptation to the special biology of SCCs, which are, for example, characterized by a specific expression pattern for different keratins (36). Furthermore, most currently used methods for the enrichment and identification of CTCs and DTCs have deficiencies in detecting tumor cells that have lost their epithelial cell features in the course of epithelial-to-mesenchymal transition (EMT; ref. 37). To circumvent this problem, Lin and colleagues used the PowerMag system to deplete leukocytes and provided evidence for the simultaneous presence of EpCAM$^+$ and EpCAM$^-$/CD45$^-$/CD45$^-$ CTC. Both types of CTC were more frequently detected in patients with cancer than in healthy probands and CTC numbers were decreased under chemotherapy (38). However, as Tinhofer and colleagues recently described, even if the expression of EpCAM (epithelial cell adhesion molecule) is downregulated, this lower EpCAM expression was strong enough to detect CTCs by flow cytometry (39).

Besides a short follow-up period with a mean follow-up time of 17 months for those patients, the lack of relapse in some patients with CTCs or DTCs might also be due to the biologic heterogeneity of CTCs/DTCs (40, 41). According to recent observations, some DTCs remain in a dormant state and may never initiate a relapse (15, 42), whereas others remain in a proliferating state or escape the dormancy control mechanisms to enter the cell cycle by still unknown conditions in the microenvironment or molecular alterations (43).

In the present study, the detection of CTCs and DTCs was significantly correlated with locoregional relapse, which might be explained by the assumption that patients with CTCs or DTCs harbor more aggressive tumors. A more provocative explanation is recirculation of tumor cells from distant sites (e.g., bone marrow) back to the site of the primary tumor, which has been reported in an experimental breast cancer model (44). Distant hematogenous metastases were less frequently observed than locoregional relapses in our present study, which might be due to the rather short follow-up period, as mentioned above.

The clinical relevance of CTC detection in patients with carcinoma was part of extensive research and encouraging results exist for an association between CTC detection and distant metastases (40, 41). According to recent observations, some DTCs remain in a dormant state and may never initiate a relapse (15, 42), whereas others remain in a proliferating state or escape the dormancy control mechanisms to enter the cell cycle by still unknown conditions in the microenvironment or molecular alterations (43).

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### Table 3. Correlations of tumor parameters and DTC

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<th>DTC-positive</th>
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<tr>
<td>Total (N = 90)</td>
<td>72 (80.0%)</td>
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<td>T1 (n = 30)</td>
<td>24 (80.0%)</td>
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<td>14 (66.7%)</td>
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<td>M1 (n = 6)</td>
<td>2 (33.3%)</td>
<td>4 (66.7%)</td>
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<td>MX (n = 11)</td>
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<tr>
<td>UICC IV (n = 31)</td>
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<td>9 (29.1%)</td>
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*Statistically significant.

### Figure 3. Forrest plot of locoregional tumor relapse by CTC, DTC, tumor size, lymph node involvement, and distant metastases; P values as indicated.
prognosis in patients with other solid tumor entities such as metastatic breast, prostate, and colorectal cancer (16–18, 45, 46). Furthermore, the clinical relevance of CTC detection was investigated in patients with various non-metastatic cancer diseases, for example, in the course of translational research programs within neoadjuvant and adjuvant treatment studies (47, 48). For patients with OSCC there are few studies investigating the dissemination of tumor cells after surgery (20, 49), but there are no studies yet investigating this issue on the impact of metastases and survival.

The results of the present study are encouraging for further investigation of the clinical relevance of CTCs and DTCs, indicating that in patients with OSCC, prediction of tumor relapse by measuring DTCs and CTCs counts may become feasible in the near future. In particular, the risk of an early locoregional relapse is difficult to assess in patients with head and neck tumors. In the past, many serum tumor markers have been evaluated for their clinical utility in HNSCC (50, 51); however, nearly all markers show an only very low sensitivity. The results of the present study indicate that the detection of CTCs/DTCs in patients with OSCC could help to fill this gap by predicting local relapse with higher sensitivity than current staging procedures. Besides further improvements in the detection of CTCs/DTCs (in particular, the detection of carcinoma cells undergoing EMT (52)), molecular characterization of CTCs/DTCs opens new perspectives to identify potential targets for individualized therapies and to use repeated CTC assessments in individual patients for treatment surveillance (53).

References

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