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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Running title: Prognostic Relevance of CTCs and DTCs in patients suffering from oral squamous cell carcinomas.

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STATEMENT OF 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. The current study indicates that CTCs/DTCs detected in OSCC-patients 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 OSCC-patients. The current findings point to the potential future utility of drugs...
targeting disseminated tumor cells in bone marrow and circulating tumor cells in blood of OSCC-patients.

Abstract: (250 words)

PURPOSE: Current staging methods for squamous cell carcinomas of the oral cavity (OSCC) need to be improved to predict the risk of individual patients. Since hematogenous tumor cell dissemination is a key event in tumor progression we assessed the prognostic significance of disseminated tumor cells (DTCs) in bone marrow (BM) and circulating tumor cells (CTCs) in peripheral blood (PB) from OSCC patients.

EXPERIMENTAL DESIGN: From 110 patients with OSCC, tumors were surgically resected (R0) without neoadjuvant therapy. The CellSearch™-system was used to enumerate CTCs. BM was aspirated from the iliac crest, and mononuclear cells (MNCs) 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 clinicopathological parameters and clinical outcome such as recurrence and death during follow up time (mean 916 days).

RESULTS: Ten/80 patients (12.5%) harbored CTCs in PB whereas in 18/90 patients (20.0%) DTCs in BM could be detected. Surprisingly, in only two patients (1.8%) CTCs and DTCs were detected simultaneously. Significant correlations could be found regarding 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 OSCC patients, 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.

Keywords: Circulating tumor cells (CTCs), Disseminated tumor cells (DTCs), bone marrow (BM), peripheral blood (PB), head and neck squamous cell carcinoma (HNSCC), Oral squamous cell carcinoma (OSCC), staging, metastasis
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 ~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 (UICC stage I-II) whereas two third 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 15-25% 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).

Over the last twenty years, the presence of disseminated tumor cells (DTCs) in bone marrow (BM) 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 BM samples from patients out of the heterogeneous group of HNSCC - including oral-, pharyngeal-, laryngeal- and skin - squamous cell carcinomas - revealed a positive correlation of DTC detection to relapse and metastases (14).

However, since repeated and sequential BM analyses are only possible in selected patients during follow-up observations, analysis of PB has become a promising alternative to investigate tumor cell dissemination (15). Especially for patients with metastatic carcinomas, the presence of CTCs in PB is associated with a worse prognosis (16-18). In only a limited number of mainly breast cancer studies the presence of DTCs in the BM 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 BM 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 radiation, (induction-)chemotherapy and/or targeted therapy for laryngeal or nasopharyngeal squamous cell carcinomas 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 radiation therapy. Therefore, it is indispensible 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 BM and CTCs in PB of patients suffering from OSCC, and to correlate these findings to 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.

**Material, 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. 110 patients (36 female, 74 male; aged 40 to 83 years; arithmetic mean 54.7 years) were investigated with histologically diagnosed squamous cell carcinoma (SCC) of the oral cavity (Table 1). Tumor size, locoregional spread and distant metastases were determined according to the TNM classification. Therefore, staging examinations included CT-scan of the head and neck and dependent on stage either x-ray of the lung and ultrasound of the abdomen for UICC1+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 HPV-Status could be examined in 54 patients and revealed positive results in 4 cases (7.4%, HPV-16) without any statistical association to clinico-pathological parameters, overall or disease specific survival and CTC/DTC findings.

Bone marrow aspirates from the iliac crest and blood samples were taken prior to 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 patient’s consent 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 circulating tumor cells (CTCs) in peripheral blood: Capturing and enumeration of circulating tumor cells (CTCs) from PB was performed with the CellSearch™-system (Veridex, Raritan, NJ, USA) (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 (Figure 1A) (16, 24).

Detection of disseminated tumor cells (DTCs) in bone marrow: Bone marrow aspiration from the upper iliac crest during primary surgery, enrichment of mononuclear cells (MNCs) by Ficoll density gradient centrifugation, preparation of cytospins 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, Huckeswagen, Germany, Figure 1B) were counted as DTCs. After incubation of two million aceton-fixed MNCs from each patient with blocking serum (Biotest, Dreieich, Germany, diluted 1:10 in PBS) for 20 minutes, incubation with A45-B/B3 (2 µg/mL in phosphate buffered saline solution with 10% AB serum) was performed. A polyclonal rabbit anti-mouse antibody (DAKO, Denmark) and a mouse alkaline phosphatase-anti-alkaline phosphatase antibody (DAKO, Denmark) 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, Germany) and subsequently the slides were mounted in Kaiser’s glycerine-gelatin (Chroma Gesellschaft GmbH, Munster, Germany). Stained slides were automatically screened for A45-B/B3-positive cells by the ACIS™-system (Chromavision, San Juan...
Capistrano, CA). 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 defective suspicious tumor cells were counted. No immunostained cells with such morphology should be detected in the corresponding negative controls (MOPC-21, Sigma-Chemical, St. Louis, MO, USA) 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 two 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 clinicopathological 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 hazard ratios 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 hazard ratio (HR) with 95% CI. Significant statements refer to p-values of 2-tailed tests that were p < 0.05.

**Results**

*Correlation of the presence of CTCs to clinicopathological parameters, relapse and survival*

In total, 110 patients were enrolled in the present study. Patients with MX stage 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, while 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 PB samples from 80 patients. CTCs could be detected in 10/80 patients (12.5%) (Figure 1A), and cell counts ranged between 1 and 14 CTCs/7.5 mL (mean: 3.2 CTCs/7.5 ml). Results of DTC and CTC detection in relation to clinico-pathological 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 (χ² test) could be found between the detection of CTCs and nodal status (pN, p=0.97) and UICC stadium (p=0.71, 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 uni- 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 three patients (3.3%) were CTC-positive in patients without relapse (n=91, p=0.0003).

No statistically significant correlation (χ² 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, Radiation therapy, concomitant Radio-/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 were 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 Odds-Ratio (OR) for CTC-positive patients was 56.06 with p=0.01.

**Correlation of the detection of DTCs to clinicopathological parameters, relapse and survival**

Eighteen of 90 patients (20%) displayed DTCs in their BM (Figure 1B). DTC numbers varied from 1 to 27/2x10^6 MNC (3.2 DTCs/2x10^6 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).

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 (8.2%) developed a pulmonary distant metastatic relapse, of whom five 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, Figure 2B, C) and additionally 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 (χ^2 test), we did not observe statistical correlations between DTC 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). Further, 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 (Figure 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 to the overall survival of the patients.

**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 two 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 pT4 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 six 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 oral squamous cell carcinoma. 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 squamous cell carcinomas 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 BM, while 10 patients (12.5%) were detected positive for CTCs in the PB, which is in accordance to literature data (14, 21). Expecting ubiquitous spread of occult tumor cells, PB should be an excellent compartment to detect these cells (“liquid biopsy”). However, similar to breast cancer patients we detected a higher frequency of BM-positive aspirates than PB-positive samples (31). The half life of CTCs in the circulation with one to two hours seems to be short (32) and hence many CTCs rapidly undergo apoptosis (33). Interestingly, there was no significant correlation between the detection of CTCs and the presence of DTCs (p=0.68) in our patient cohort, suggesting that both analyses provide, therefore, complementary results.

In the present study, our follow up evaluation and statistical analysis demonstrated a significant correlation between positivity for DTCs or CTCs and the subsequent development
of recurrent disease. But we found no further 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 HNSCC patients. Our present study is, therefore, the first report on the prognostic value of CTCs and DTCs in a homogeneous group of OSCC patients, 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 co-morbidities.

Finally, the detection of DTCs and CTCs was significantly associated with the presence of distant metastases. Since additionally, postoperative treatment modalities such as radiotherapy or concomitant radio-/chemotherapy revealed no significant impact on locoregional recurrence, this indicates that patients positive for tumor cells in the BM or PB 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 adaption to the special biology of squamous cell carcinomas, 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) (37). To circumvent this problem, Lin et al. (2013) used the PowerMag system to deplete leukocytes and provided evidence for the simultaneous presence of EpCAM+CD45- and EpCAM/CD45- CTC. Both types of CTC were more frequently detected in cancer patients than in healthy probands and CTC numbers were decreased under chemotherapy (38). However, as Tinhofer et al. 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 biological 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 to locoregional relapse, which might be explained by the assumption that patients with CTCs or DTCs harbor more aggressive tumors. A more provocative explanation is re-circulation 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 carcinoma patients was part of extensive research and encouraging results exist for an association between CTC detection and 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 OSCC patients 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 OSCC patients 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).

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References


Legend to Tables

Table 1: Patient Characteristics

Table 2: Correlations of tumor parameters and CTCs

Table 3: Correlations of tumor parameters and DTCs

Legend to Figures

Figure 1: Presentation of selected circulating tumor cells (CTCs) detected in peripheral blood (A) and disseminated tumor cells (DTCs) detected in bone marrow (B). A: Image gallery presenting 5 circulating tumor cells (CTCs) detected by the CellSearch™-system: DAPI (4', 6-Diamidino-2-Phenylindole)- and keratin (PE: phycoerythrin)-positive, but CD45 (APC, allophycocyanin)-negative cell with a diameter of at least 4 µm. B: Nucleated cells that express keratins as demonstrated by positivity for the pan anti-keratin antibody A45-B/B3 (red). Counterstaining of nuclei by hemalaun (blue).

Figure 2: Correlation of CTC and DTC detection with time to locoregional or metastatic relapse. A: Time to locoregional relapse by circulating tumor cells (CTC); p<0.001. B: Time to locoregional relapse by disseminated tumor cells (DTC); p<0.001. C: Time to metastatic relapse by disseminated tumor cells (DTC); p<0.001

Figure 3: Forrest plot of locoregional tumor relapse by CTC, DTC, tumor size, lymph node involvement and distant metastases, p-values as indicated.
Figure 1
Figure 2

(A) Probability of event over time for two groups: negative [0] and positive [1].

- Number at risk: 54, 33, 13, 11, 3, 2, 2
- Time to event (years): 0, 1, 2, 3, 4, 5, 6
- p-value: <0.001

(B) Probability of event over time for two groups: negative [0] and positive [1].

- Number at risk: 55, 40, 25, 23, 10, 6, 4
- Time to event (years): 0, 1, 2, 3, 4, 5, 6
- p-value: <0.001

(C) Probability of event over time for two groups: negative [0] and positive [1].

- Number at risk: 53, 37, 25, 22, 10, 6, 4
- Time to event (years): 0, 1, 2, 3, 4, 5, 6
- p-value: <0.001
Figure 3

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Relapse less frequent → relapse more frequent

[Diagram showing relationships between different stages and OR, CI, p-values]
## Tables

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<td>21 (19.1%)</td>
</tr>
<tr>
<td>Radiation+ concurrent chemotherapy</td>
<td>44 (40.0%)</td>
</tr>
</tbody>
</table>

**Table 1:** Patient Characteristics
<table>
<thead>
<tr>
<th>Variables</th>
<th>CTC negative</th>
<th>CTC positive</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n=80)</td>
<td>70 (87.5%)</td>
<td>10 (12.5%)</td>
<td>p=0.04*</td>
</tr>
<tr>
<td>T1 (n=24)</td>
<td>23 (95.8%)</td>
<td>1 (4.2%)</td>
<td></td>
</tr>
<tr>
<td>T2-T4 (n=56)</td>
<td>47 (83.9%)</td>
<td>9 (16.1%)</td>
<td></td>
</tr>
<tr>
<td>N0 (n=38)</td>
<td>33 (86.8%)</td>
<td>5 (13.2%)</td>
<td>p=0.97</td>
</tr>
<tr>
<td>N1 (n=20)</td>
<td>17 (85.0%)</td>
<td>3 (15.0%)</td>
<td></td>
</tr>
<tr>
<td>N2a-c (n=21)</td>
<td>19 (90.5%)</td>
<td>2 (9.5%)</td>
<td></td>
</tr>
<tr>
<td>N3 (n=1)</td>
<td>1 (100%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>M0 (n=67)</td>
<td>61 (91.0%)</td>
<td>6 (9.0%)</td>
<td>P=0.004*</td>
</tr>
<tr>
<td>M1 (n=5)</td>
<td>1 (20.0%)</td>
<td>4 (80.0%)</td>
<td></td>
</tr>
<tr>
<td>MX (n=8)</td>
<td>8 (100%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>UICC I (n=15)</td>
<td>14 (93.3%)</td>
<td>1 (6.7%)</td>
<td>p=0.71</td>
</tr>
<tr>
<td>UICC II (n=10)</td>
<td>9 (90.0%)</td>
<td>1 (10.0%)</td>
<td></td>
</tr>
<tr>
<td>UICC III (n=27)</td>
<td>22 (81.5%)</td>
<td>5 (18.5%)</td>
<td></td>
</tr>
<tr>
<td>UICC IV (n=28)</td>
<td>25 (89.3%)</td>
<td>3 (10.7%)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2:** Correlations of tumor parameters and CTC

* = statistically significant
<table>
<thead>
<tr>
<th>Variables</th>
<th>DTC negative</th>
<th>DTC positive</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n=90)</td>
<td>72 (80.0%)</td>
<td>18 (20.0%)</td>
<td>p=0.16</td>
</tr>
<tr>
<td>T1 (n=30)</td>
<td>24 (80.0%)</td>
<td>6 (20.0%)</td>
<td></td>
</tr>
<tr>
<td>T2 (n=25)</td>
<td>21 (84.0%)</td>
<td>4 (16%)</td>
<td></td>
</tr>
<tr>
<td>T3 (n=14)</td>
<td>13 (92.9%)</td>
<td>1 (7.1%)</td>
<td></td>
</tr>
<tr>
<td>T4 (n=21)</td>
<td>14 (66.7%)</td>
<td>7 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>N0 (n=44)</td>
<td>37 (84.1%)</td>
<td>7 (15.9%)</td>
<td>p=0.02*</td>
</tr>
<tr>
<td>N1 (n=22)</td>
<td>20 (90.9%)</td>
<td>2 (9.1%)</td>
<td></td>
</tr>
<tr>
<td>N2a-c (n=21)</td>
<td>12 (57.1%)</td>
<td>9 (42.9%)</td>
<td></td>
</tr>
<tr>
<td>N3 (n=3)</td>
<td>3 (100%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>M0 (n=73)</td>
<td>60 (82.2%)</td>
<td>13 (7.8%)</td>
<td>p&lt;0.01*</td>
</tr>
<tr>
<td>M1 (n=6)</td>
<td>2 (33.3%)</td>
<td>4 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>MX (n=11)</td>
<td>10 (90.9%)</td>
<td>1 (9.1%)</td>
<td></td>
</tr>
<tr>
<td>UICC I (n=22)</td>
<td>18 (81.9%)</td>
<td>4 (18.1%)</td>
<td>p=0.18</td>
</tr>
<tr>
<td>UICC II (n=10)</td>
<td>10 (100%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UICC III (n=27)</td>
<td>22 (81.5%)</td>
<td>5 (18.5%)</td>
<td></td>
</tr>
<tr>
<td>UICC IV (n=31)</td>
<td>22 (70.9%)</td>
<td>9 (29.1%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Correlations of tumor parameters and DTC

*= statistically significant
Clinical Cancer Research

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

Alexander Grobe, Marco Blessmann, Henning Hanken, et al.

Clin Cancer Res  Published OnlineFirst November 11, 2013.

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