Evaluation of incidence, significance, and prognostic role of circulating tumor microemboli and transforming growth factor-β receptor I in head and neck cancer

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Abstract
Background: Circulating tumor microemboli (CTM) are clusters of circulating tumor cells (CTCs), involved in metastasis, as also transforming growth factor-β (TGF-β). The purpose of this study was to verify their role in progression-free survival (PFS).

Methods: Blood from patients with locally advanced head and neck squamous cell carcinoma (HNSCC; n = 53) was analyzed in 2 moments. TGF-β receptor I (TGF-βRI) expression was evaluated by immunocytochemistry.

Results: Comparing CTM1 (baseline) with CTM2 (first follow-up), patients with CTM1-positive disease who became CTM2-negative were classified as favorable (PFS 20 months). Patients with unfavorable evolution (CTM1-negative/CTM2-positive), had PFS of 17.5 months. Patients always CTM-negative showed PFS of 22.4 months, those always positive, 4.7 months (P < .001). The TGF-βRI expression in the first follow-up correlated with poor PFS (12 ± 26 months; P = .007), being an independent prognostic factor (hazard ratio [HR] = 6.088; P = .033).

Conclusion: CTM1/2, TGF-βRI expression, and unfavorable CTM kinetics may represent poor prognosis in locally advanced HNSCC.

Keywords
circulating tumor microemboli, head and neck cancer, isolated circulating tumor cells, poor prognosis, transforming growth factor-β receptor I (TGF-βRI)

1 INTRODUCTION

Metastasis mechanism can be considered, initially, as an inefficient process. A significant amount of cancer cells can circulate through the bloodstream in patients with cancer, but very few of these can develop into metastases and characterize a patient with metastatic disease.4,5 However, when metastasis succeeds, the management of every single patient is in fact complex and expected survival is very limited,Justifying the efforts in understanding it. It is currently known that mechanical factors are important in cancer cell spreading, as well as a positive interaction between the cancer cell surface proteins and receptors and the microenvironment of a specific organ.6 This process can be prevented by the immune system, in
reaction against tumor-specific or associated agents.\textsuperscript{7} Meanwhile, despite all efforts to eliminate tumor cells, the immune system can be orchestrated by cytokines to induce cancer-related inflammation.\textsuperscript{8,9} In primary carcinomas, growth factors and cytokines secreted by immune and tumor cells contribute to tumor development in several key functions (motility, invasion, and dissemination) by activation of a process called epithelial-mesenchymal transition (EMT).\textsuperscript{10} Epithelial tumor cells gain migratory abilities by the EMT process, which is also considered to be responsible for drug resistance during anticancer treatment.\textsuperscript{6} Some molecules are important in this process, such as transforming growth factor-β (TGF-β) and matrix metalloproteinase 2 (MMP-2).

The TGF-β is one of the most studied EMT signaling pathways. It is a multifunctional cytokine that has 3 receptors to bind to ligand, TGF-β receptors I, II, and III (TGF-βRI, TGF-βRII, and TGF-βRIII). The signal is triggered after binding of the TGF-β ligand to TβR-II or to the accessory receptor, TβR-III, which transfers TGFβ to TβR-II. After the transfer of the cytokine, this recruits and phosphorylates the signaling type I TGFβ receptor (TβR-I). The TβR-I acts downstream the TβR-II and determines the specificity of intracellular signals by phosphorylating transcriptional cytoplasmic factors, major of the signaling pathway Smad2 and Smad3.\textsuperscript{11–13} In addition, it was shown\textsuperscript{3} that platelets secrete TGFβ1, by activating βI/Smad signaling pathway in tumor cells and that megakaryocyte/platelet-specific deficiency of TGF-βI is able to inhibit metastasis formation in lungs of mice. The MMP-2, on the other hand, is a gelatin cleaving metalloproteinase – called gelatinase – that also binds to fibrinogen and targets several chemokines, such as TGF-β, which is activated from its latent form by MMP-2 and MMP-9.\textsuperscript{14} The MMP-2 can also be regulated by TGF-β, and both together lead to the initiation of the cascade of various events linked to tumor proliferation, survival, and invasion.\textsuperscript{15,16}

It seems that EMT can allow cancer cells to detach from the primary tumor and enter the blood circulation in the form of CTCs.\textsuperscript{6} Much has been done recently to understand the biology of CTCs and to make these cells a new approach in order to monitor the evolution of patients with cancer.\textsuperscript{16} The present knowledge makes CTC studies important tools in clinical practice, once they can be of predictive and prognostic significance in many cancer types, such as breast, ovary, lung, colon, and prostate.\textsuperscript{17–21} Another aspect concerns CTCs aggregated in clusters, or CTMs, which is defined as clusters of 3 or more cancer cells.\textsuperscript{2} The CTMs are more prone to evade successfully immune mechanisms than iCTCs, because they are complex structures. There are few studies showing the prognostic role of these structures in some tumor types, such as colon, prostate, kidney, and nonsmall cell lung cancer.\textsuperscript{1,22–27} Recently, Hou et al\textsuperscript{1} (2012) showed worst prognosis for patients with small cell lung cancer (SCLC) positive for CTMs in comparison with those with iCTCs, not only for PFS, but also for overall survival.

Here, we present our attempt to characterize the molecular profiling of CTMs isolated from patients with head and neck cancer, looking at 2 proteins directly involved in EMT, TGF-βRI and MMP-2, in these cell clusters and in iCTCs. The purpose of this study was to verify the role of CTMs in disease progression, as also the involvement of TGF-βR and MMP-2 in this scenario, by their observation in iCTCs and in CTMs.

## 2 | MATERIALS AND METHODS

### 2.1 | Patient samples

This was a single institution study, held at A.C. Camargo Cancer Center, São Paulo, Brazil. Patients with locally advanced head and neck squamous cell carcinoma (HNSCC) candidates to a curative intent treatment (adjuvant chemoradiotherapy, definitive radiotherapy [RT] concurrent with chemotherapy or cetuximab, or induction chemotherapy followed by RT concurrent with chemotherapy or cetuximab) were prospectively enrolled for iCTCs and CTM analysis (53 patients, institution ethic review board number: 1777/13). All patients were offered written informed consent for participation in the study, and all 53 of the patients accepted to participate without restrictions. The aim was to identify the prevalence of iCTCs and CTMs, their molecular profile, and to analyze frequencies and relation with demographic characteristics and PFS. Patients were included from January 2014 to June 2016. Patients with a second primary tumor and with loss of follow-up > 1 year were excluded from the final analysis. Blood samples were collected in 2 moments: before the beginning of treatment (definitive or adjuvant; baseline) and after approximately 3 cycles of induction chemotherapy or after completion of RT with chemotherapy or cetuximab, with an average of 3 months’ difference between the 2 samples (first follow-up). Definitive treatment involves up-front RT concurrent with chemotherapy or cetuximab or induction chemotherapy with taxane, cisplatin, and 5-fluorouracil regimen for 3 cycles followed by RT plus chemotherapy or cetuximab. Adjuvant treatment after surgery consists of concurrent chemotherapy and RT.

### 2.2 | Circulating tumor cells and circulating tumor microemboli analysis

The analysis of CTCs and CTMs were made by the ISET (Isolation by Size of Epithelial Tumor Cells; Rarecells, France), which consists in a direct method for isolation of tumor cells by filtration in a polycarbonate membrane with calibrated pores with 8-µm diameter. This method is based on the premise that cancer cells are larger than leukocytes
that usually are smaller than 8 µm. Briefly, 8 mL of blood were diluted in ISET® buffer, then transferred to the ISET® block coupled to the polycarbonate membrane, which contains 10 spots with millions of cylindrical pores of 8 µm. The samples were filtered through the ISET device. Most leukocytes were eliminated by filtration. Membranes were preserved at −20°C until immunostaining analysis. Fixed intact CTCs on ISET spots were stained by immunocytochemistry (ICC) and counterstained with hematoxylin. The CTCs were counted in 4 spots of the membrane and quantified in 1 mL of blood.26 We used blood of healthy donors as negative control and healthy blood spiked with SCC-9 (squamous cell carcinoma) cell line as positive control.

2.3 | Circulating tumor cell immunostaining

To analyze the molecular profile of CTMs and iCTCS, we performed an ICC assay using a protocol previously described.28 The following antibodies were chosen: anti-TGF-βRI (Polyclonal, Cusabio, China, 1:100, code: CSB-PA061850), and anti-MMP-2 (Polyclonal, Cusabio, China, 1:100, code: CSB-PA014666GA01HU).

For ICC reactions, we used A-549 and PC3 cell lines, which, according to the Human Protein Atlas (http://www.proteinatlas.org/29 express TGF-βRI and MMP-2, respectively. The cells were “spiked” in a healthy donor blood and filtered on ISET (Figure 1A,D). We used the same cell-line, omitting the primary antibody, as negative control for ICC, to ensure the exclusion of cross-reactivity. To exclude leukocytes of our tumor cells counting, we used anti-CD45 antibody, to ensure the exclusion of cross-reactivity. To exclude leukocytes of our tumor cells counting, we used anti-CD45 antibody, as negative control for ICC, to ensure the exclusion of cross-reactivity.

2.4 | Statistical analysis

The baseline patient characteristics were expressed as absolute and relative frequencies for qualitative variables. The determination of 2 groups of observations with respect to a simple cut-point was estimate using the maximum of the standardized log-rank statistic proposed by Lausen and Schumacher,30 in 1992. The Kaplan-Meier estimator of the survival function was considered for survival analysis and the log-rank test was applied to compare the survival distribution between groups. The Cox semiparametric proportional hazards model31 was used to describe the relationship between survival and progression times and covariate defined with respect to a cut-point. We assessed the proportionally assumption on the so-called Schoenfeld residuals.32,33 In all cases, there is evidence that covariates have a constant effect over time.

Survival analysis for PFS was based on the date of first CTC collection and the first progression (considered as PFS) after collection. The CTM quantification and the molecular analysis, as also iCTCs molecular profile, were evaluated in correlation with the clinical evolution using the chi-square test. The statistical significance level was set to 0.05. Data was processed using the SPSS for Windows software, version 15.0.

3 | RESULTS

3.1 | Demographic and clinical variables

Information about the demographic characteristics is summarized in Table 1. In the 53 patients enrolled, the median age at diagnosis was 60.4 years (range 42.2-76.8 years), with a predominance of male sex (n = 44; 83.0%). All patients had non-metastatic locally advanced disease (39.6% of patients with T3 or T4 disease, and 58.5% with N2 or N3 disease).

At the time of analysis, 18 patients (34%) had progressive disease by the established criteria (Table 1). We evaluated the relation between clinical variables and PFS. As expected, we found a correlation between T classification status and PFS, as patients with T3/T4 classifications had poor PFS (16.6 months) compared to T0/T1/T2 classifications (23.8 months; P = .046).

3.2 | Circulating tumor cell count analysis

With a median follow-up of 15.5 months, the CTC detection rate in locally advanced HNSCC using the ISET method was 92.5% at baseline (49/53) and 93.8% in the first follow-up (30/32; Figure 1B). The median CTC count was 3.0 CTCs/mL (range 0.0-8.0) at baseline and 1 CTC/mL in the first follow-up (range 0.0-12.0) and CTMs were positive in 15 patients (28.3%) at baseline and 7 (23.3%) in the first follow-up.

The CTC counts were evaluated considering patients above and equal or below the median. There was a numeric difference without statistical significance for patients with CTC levels above the median with worse PFS in comparison to those below the median at baseline (17.6 vs 23.7 months; P = .13) and in the first follow-up (20.5 vs 26 months; P = .26; Figure 2A,B). There was a clear correlation between CTC counts and the presence of CTMs, with a higher prevalence of CTMs in patients with CTC counts above median at baseline (P < .001). In the first follow-up, this relation was present but not statistically significant (P = .43). There was also correlation between the presence of CTMs at baseline (CTM1) and development of distant metastasis, because among the 9 patients with metastasis, 5 (55.6%), were CTM1-positive (P = .046). For CTMs in the first follow-up (CTM2), this correlation was not found (P = .08).
3.3 | Progression-free survival analysis

The mean PFS of the entire population was 12.23 months (range 0.13-31 months). Among 15 patients with CTMs at baseline, 6 (40%) showed disease progression, in comparison to 9 patients (60%) without CTMs. Although, without statistical significance, there was a relationship between CTM presence at baseline and PFS, as patients with CTMs had a PFS of 18.8 months in comparison to 23 months for those without CTMs \((P = .14; \text{Figure 2C})\). In the first follow-up analysis, this relation was clearer, with 4 disease progressions among 7 patients with CTMs (57%), with a median PFS of 13.22 months versus 21.8 months for patients without CTMs \((P = .19; \text{Figure 2D})\).

3.4 | Kinetics

Assessment of the presence of CTMs (Figure 3A,C) at 2 serial collections allowed an analysis of CTM kinetics, as made in our previous work with CTC counts in metastatic colorectal cancer.\(^{34}\) Comparing the presence of CTMs at baseline (CTM1) with their presence at first follow-up (CTM2), patients with CTM-positive at baseline who became CTM-negative in the first follow-up were classified with a favorable evolution and had a median PFS of 20 months. This was higher than the PFS of patients with an unfavorable evolution (CTM1-negative and CTM2-positive), with a median PFS of 17.5 months. Patients always negative for the presence of CTMs showed the best PFS of 22.42 months. Patients always positive for CTM presence showed the worst PFS of 4.7 months \((P < .001; \text{see Figure 4})\).

3.5 | Molecular characteristics of circulating tumor cells/circulating tumor microemboli

Not all patients have samples tested for TGF-βRI and MMP-2 at baseline and first follow-up. Among the 53 patients included, 24 patients had TGF-βRI and 38 patients had MMP-2 protein expression analyzed on iCTCs/CTMs at baseline. At first follow-up, among 32 patients, 21 and 24 were analyzed for TGF-βRI and MMP-2, respectively. The number of ICC assays available to evaluate the protein expression of TGF-βRI and MMP-2 were reduced due to some technical reasons (absence of additional spot to make the analysis or no CTCs found in the spot analyzed). At baseline, 8 patients (33.3%) were positive for TGF-βRI expression (5 on iCTCs; Figure 1C; and 3 in CTMs; Figure 3B). For MMP-2, 24 (63.1%) were positive (24 in iCTCs; Figure 1F; and 3 in CTMs; Figure 3D). In the first follow-up, 7 patients (33.3%) had CTCs positive for TGF-βRI (1 in CTMs and 7 in iCTCs), and 14 patients (58.3%) had CTCs positive for MMP-2 (2 in CTMs and 14 in iCTCs). Among patients with CTMs, there was a clear influence of TGF-βRI...
expression at baseline and poor PFS (13.7 months vs 21.82 months; \( P = .17 \); Figure 5A), although without statistical significance. In the first follow-up, this correlation was more conspicuous, as the only patient positive for TGF-\( \beta \)-RI in CTMs had a PFS of 4.6 months versus 23 months for those with CTM-negative for TGF-\( \beta \)-RI expression (\( P < .001 \)). The expression of TGF-\( \beta \)-RI in iCTCs in the first follow-up also correlated with poor PFS (12 vs 26 months; \( P = .007 \); Figure 5B). The combined analysis of the expression of TGF-\( \beta \)-RI in CTCs at first follow-up (iCTCs and CTMs) correlated with poor PFS (12 vs 26 months; \( P = .007 \)). No relation between MMP-2 and PFS in iCTCs or CTMs at baseline or in the first follow-up was observed.

### 3.6 Multivariate analysis

A Cox proportional hazards model was fitted to the data to evaluate the relationship between the independent variables of interest over time until the occurrence of progression. The assumption of proportionality was assessed using the scaled Schoenfeld residuals. In all cases, there was evidence that the covariate has a constant effect over time. By Cox proportional hazard model, we evaluated CTMs, TGF-\( \beta \)-RI in iCTCs, and TGF-\( \beta \)-RI in iCTCs/CTMs at first follow-up as covariates. The expression of TGF-\( \beta \)-RI in iCTCs at first follow-up (iCTCs and CTMs) correlated with poor PFS (12 vs 26 months; \( P = .007 \)). No relation between MMP-2 and PFS in iCTCs or CTMs at baseline or in the first follow-up was observed.

### 4 DISCUSSION

The presence of CTMs and their relation to tumor progression have been observed in some studies, showing their possible

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**TABLE 1** Patients with locally advanced head and neck squamous cell carcinoma demographic and clinicopathological characteristics

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(Continues)
contribution to poor prognosis. However, the understanding of the structure of these CTMs is still under investigation. A major challenge in CTM analysis is that many CTC isolation methods disrupt cell-cell contact, leading to the breakage of the cell cluster. The usage of techniques based on cell size, such as ISET, to isolate CTCs makes it possible to distinguish between a single cell and the cell clusters.26,27

To our knowledge, our study is the first to show a high CTC detection rate (92.5%) in head and neck cancer and the first to try to explore the presence of invasion proteins in these cells and in CTMs. There are some studies showing detection rates of CTCs varying from 15% to 43% in locally advanced head and neck cancer.35–38 These studies used methods based on antibody selection (cytokeratin and epithelial cell adhesion molecule) to isolate CTCs. However, it is demonstrated that CTCs from patients with head and neck cancer do not express cytokeratin.39 This explains why we could detect as much CTCs in these patients as we did, as we used a method that isolates CTCs independently of antibody selection.

The CTMs are greater in size than CTCs. Considering the simple mechanics of perfusion of organs, CTMs are more prone to be arrested in the vascular bed than the small CTCs. Hou et al1 (2012) observed the proliferation status of CTMs by Ki-67 staining. All patients with SCLC positive for CTM had a negative Ki67 staining, even those patients with iCTCs positive for this proliferation marker. Apoptotic status of CTMs was also verified, with similar results, no CTM was positive for antiapoptotic or apoptotic protein staining. The same impact on worse prognosis of CTMs seen for PFS in those patients with SCLC was demonstrated in our study. In addition, we analyzed the CTM kinetics and observed that a cellular clonal selection seems to occur, because the change from favorable into unfavorable profile correlated with poor PFS, indicating that the treatment was not effective in eliminating resistant clones (P < .001). Our findings are also in agreement with a recent study published by Long et al2 (2016) with 128 patients with metastatic melanoma. They characterized CTC phenotype using melanoma markers and, unlike our study, they counted the CTMs. The CTCs were detected in 109 of 128 of their patients (85%), 44 of 128 (34%) with 2-6 CTMs, and 65 of 128 (51%) with 4-9 iCTCs. The overall survival was significantly worse in patients with

FIGURE 2 Progression-free survival (PFS) of patients with locally advanced head and neck squamous cell carcinoma in relation to circulating tumor cells (CTCs) and circulating tumor microemboli (CTM). These cells were collected before the beginning of chemotherapy and after 3 months of treatment. The CTC cutoff was defined as described in the Methods section. A, Baseline analysis of CTCs: ≤3 CTCs/mL: median PFS of 23.7 months vs >3 CTCs/mL: 17.6 months (P = .13). B, First follow-up analysis of CTCs: ≤1 CTCs/mL: median PFS of 26.0 months vs >1 CTCs/mL: 20.5 months (P = .26). C, Baseline analysis of CTM. Patients with absence of CTMs had median PFS of 23.0 months versus 18.8 months for those with presence of CTMs (P = .14). D, First follow-up analysis of CTM. Patients with absence of CTMs had median PFS of 21.8 months vs 13.22 months for those with presence of CTMs (P = .19).
CTMs, independently of the therapeutic strategies, indicating, as in our findings, that CTMs may contain resistant cell clones. Other studies observed the detection of CTMs when analyzing CTCs in colorectal, prostate, renal, and lung cancers, showing that CTCs in clusters are not a rare event, but really need to be better explored.

In our study, we also assessed TGF-βRI and MMP-2 status. The choice of these 2 EMT markers was based on the central role they play on cellular differentiation, invasion, and metastasis development, and the possible implication of the positivity for these factors in cancer prognosis. The evaluation for invasiveness profile with TGF-βRI and MMP-2 can correlate with prognosis, as seen in preclinical models using the EMT phenotype in genomic studies. Some recent works include the evaluation of the EMT phenotype in drug resistance, as exemplified by preclinical data of development of resistance to tyrosine-kinase inhibitors in lung cancer. These examples establish an inherent relationship among TGF-β, MMP-2, and the metastatic process.

Recently, Labelle et al. (2011) who were working with megakaryocytes knockout to TGF-β1 expression (model of TGF-β1 floxed/platelet factor 4 cre mouse) showed that CTMs, independently of the therapeutic strategies, indicating, as in our findings, that CTMs may contain resistant cell clones. Other studies observed the detection of CTMs when analyzing CTCs in colorectal, prostate, renal, and lung cancers, showing that CTCs in clusters are not a rare event, but really need to be better explored.

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**FIGURE 3** Circulating tumor microemboli (CTM) from patients with locally advanced head and neck squamous cell carcinoma. A, The CTMs negative for transforming growth factor-β receptor I (TGF-βRI) expression, visualized with hematoxylin-eosin stain (original magnification ×40). B, The CTMs positive for TGF-βRI expression, visualized with diaminobenzidine (DAB) (original magnification ×40). C, The CTMs negative for matrix metalloproteinase (MMP-2) expression, visualized with hematoxylin-eosin stain (original magnification ×40). D, The CTMs positive for MMP-2 expression, stained with DAB (original magnification ×40). Photomicrographs were taken by a light microscope (Research System Microscope BX61; Olympus, Tokyo, Japan) coupled to a digital camera (SC100; Olympus).

**FIGURE 4** Progression-free survival (PFS) in relation to kinetics of circulating tumor microemboli (CTM) from patients with locally advanced head and neck squamous cell carcinoma. Dashed-dotted line: patients with favorable kinetics and prognosis (CTM always negative; PFS = 22.42 months). Dashed line: patients who changed from unfavorable (CTM-positive) to favorable (CTM-negative), PFS = 20.0 months. Continuous line: patients who changed from favorable (CTM-negative) to unfavorable (CTM-positive), PFS = 17.5 months. Dotted line: patients with unfavorable kinetics and prognosis (CTM always positive; PFS = 4.7 months; \( P < .001 \)).
metastases in those mice were diminished. These results suggest that tumor cell behavior is altered due to platelet activation, as the absence of TGF-β1 in platelets interfered in tumor cell extravasation to the lungs. These authors propose that platelets probably provide TGF-β1 to CTCs, allowing them to gain a more invasive, mesenchymal-like phenotype that helps them to extravasate.

The TGF-β can act in many different ways, favoring tumor metastasis. The TGF-β stimulates reactive oxygen species production, which leads to downstream signaling pathways (e.g., epidermal growth factor receptor, Src, SMADs, and mitogen-activated protein kinase family) resulting in expression of profibrotic genes (e.g., connective tissue growth factor, TGF-β1). It is demonstrated that TGF-β overproduction precedes tumor formation and prepares a favorable microenvironment for cancer cells. The TGF-β pathway also plays a protumoral role by activating angiogenesis, as it acts in an autocrine/paracrine way with other signaling cascades, such as vascular endothelial growth factor, basic fibroblast growth factor, platelet-derived growth factor, angiopoietin, and Notch. The TGF-β has both proangiogenic and antiangiogenic properties, depending on its expression level. Lower levels contribute to angiogenesis indirectly by upregulating expression/activity of angiogenic factors (vascular endothelial growth factor, basic fibroblast growth factor). Higher levels of TGF-β stimulate basement membrane reformation, recruitment of smooth muscle cells, and inhibition of endothelial cell growth.

Although not all patients have samples tested for technical reasons, those who were tested allowed us to conclude that the TGF-βRI expression in the first follow-up in iCTCs and/or CTMs is determinant for poor PFS, as showed by multivariate analysis (HR = 7.1; P = .02). At baseline, this expression was important, but statistical significance was found only in the first follow-up. We have 2 hypotheses to explain this: (1) maybe the most important when evaluating CTCs counts, is to use them after the first-line treatment, as shown by Inhestern et al (2015) in head and neck cancer; and (2) it is possible that, after the first-line treatment, a cell clone selection occurs with more resistant cells arising from the primary tumor. Our results and those from Labelle et al (2011) strongly suggest that TGF-βRI inhibitor must be tested in clinical trials. There are many preclinical and clinical trials ongoing, and we believe that CTM and expression of TGF-βRI in CTCs/CTMs can be a useful tool to predict treatment response in locally advanced HNSCC.

![FIGURE 5](image) Progression-free survival (PFS) of patients with locally advanced head and neck squamous cell carcinoma in relation to transforming growth factor-β receptor I (TGF-βRI) expression in isolated circulating tumor cells (iCTCs) and circulating tumor microemboli (CTM). These cells were collected before the beginning of chemotherapy and after 3 months of treatment. A, The TGF-βRI expression in iCTCs and CTM at baseline: median PFS of 18.0 months versus 21.5 months for negative expression (P = .17). B, The TGF-βRI expression in iCTCs and CTMs in the first follow-up: median PFS of 12.0 months versus 26.0 months for negative expression (P = .007).

![TABLE 2](image) Multivariate regression analysis of factors associated with disease progression, showing expression of transforming growth factor-β receptor I and the presence of circulating tumor microemboli at first follow-up as independent prognostic factors

<table>
<thead>
<tr>
<th>Variables</th>
<th>HR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-βRI in iCTCs and CTM (first follow-up)</td>
<td>7.1</td>
<td>1.35-37.25</td>
<td>.02</td>
</tr>
<tr>
<td>CTM (first follow-up)</td>
<td>1.6</td>
<td>0.32-8.25</td>
<td>.057</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; CTM, circulating tumor microemboli; HR, hazard ratio; iCTCs, isolated circulating tumor cells; TGF-βRI, transforming growth factor β receptor I.
In conclusion, our data are consistent with previous studies, demonstrating the worse prognosis for patients who were positive for CTM in their circulation in locally advanced HNSCC. We also found an important marker of invasion, TGF-βRII, expressed in iCTCs and CTMs and correlated with worse PFS, which suggest that more studies need to be made to validate our data for clinical use.

**NOVELTY AND IMPACT OF THE WORK**

The presence of circulating tumor microemboli (CTM) and their relation with tumor progression have been described in some studies, with a correlation to poor prognosis.\(^1\)\(^2\) However, the understanding of the structure of these CTMs is still under investigation. To our knowledge, our study is the first to show a high circulating tumor cell (CTC) detection rate (92.5%) in patients with head and neck cancer and to explore the presence of invasion proteins in these cells and in CTMs. The transforming growth factor β receptor I (TGF-βRI) expression in the first follow-up in isolated CTCs (iCTCs) and/or CTMs was determinant for poor progression-free survival (PFS), as showed by multivariate analysis (hazard ratio [HR] = 7.1; \(P = .02\)). At baseline, this expression was important, but statistical significance was found only in the first follow-up. Our results and those from Labelle et al\(^3\) (2011) strongly suggest that TGF-βRI inhibition may have a role in cancer treatment and should be tested in clinical trials.

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**DECLARATIONS**

The study was approved by the local Research and Ethics Committee (CEP – Comitê de Ética e Pesquisa). The consent was registered under the number 1777/13. All participants were offered written informed consent by the researchers, with full approval of all subjects.

**CONFLICT OF INTEREST**

The authors have declared no conflicts of interest.

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**REFERENCES**


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