UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

NATHÁLIA MARTINS LOPES

Isolation and characterization of oral cancer stem cells in primary lesions and peripheral blood: role of the phenotypic plasticity in the process of metastasis

Isolamento e caracterização de células-tronco de carcinoma epidermóide de boca em tecido fresco e sangue periférico: papel da plasticidade fenotípica no processo de metástase

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“A persistência é o caminho do êxito.”

Charles Chaplin

ABSTRACT

Isolation and characterization of oral cancer stem cells in primary lesions and peripheral blood: role of the phenotypic plasticity in the process of metastasis

Recent evidence shows that mechanisms of tumor recurrence and metastasis, as well as failure to treat oral squamous cell carcinoma (OSCC), are due, among other factors, to the patterns of cellular heterogeneity present in tumors that can be explained by the model of cancer stem cells (CSC). It is also known that there is a link between CSC and the epithelial-mesenchymal transition (EMT) process, which explains the greater capacity of migration and metastatic potential of CSCs compared to the other tumor cells. In addition, when tumor cells detach from the primary site of the tumor and enter the peripheral circulation, they are called circulating tumor cells (CTCs), capable of depositing in the lymph nodes and other organs, where they can proliferate and originate metastatic tumors. In this context, the present study includes both an experimental approach and a systematic literature review. The experimental approach aimed to quantify and characterize the CSC in fresh tumor tissue from primary OSCC lesions, associating biological properties related to the stem tumor and TEM phenotypes with the invasive and metastatic behavior of the OSCC. For this purpose, the expression levels of CD44 and ESA were evaluated by flow cytometry in fresh tissue samples in order to identify the CSC subpopulation as well as its $CD44^{+}ESA^{-}$ (TEM/mesenchymal) and $CD44^{+}ESA^{+}$ (epithelial) subfractions, correlating these data to clinicopathological parameters. The results showed that the $CD44^{+}ESA^{-}$ (mesenchymal) subpopulation was significantly associated with the degree of tumor differentiation and alcohol consumption, while the $CD44^{+}ESA^{+}$ (epithelial) subpopulation was directly correlated with the perineural invasion and regional lymph node compromising. Complementarily, considering the relevance of CTCs as minimally invasive biomarkers of cancer progression, as well as their phenotypic plasticity, a systematic review of CTC detection methodologies specifically in OSCC was performed.

Keywords: Oral squamous cell carcinoma, cancer stem cells, epithelial-mesenchymal transition, phenotypical plasticity, circulating tumor cells, flow cytometry.

RESUMO

Evidências recentes mostram que mecanismos de recorrência tumoral e metástase, assim como a falha do tratamento do carcinoma epidermóide de boca (CEB), se devem, entre outros fatores, aos padrões de heterogeneidade celular presente nos tumores que pode ser explicado pelo modelo de células-tronco de câncer (CSC, do inglês *cancer stem cells*). Sabe-se, ainda, que há uma relação entre as CSC e o processo de transição epitélio-mesenquimal (TEM), que explica a maior capacidade de migração e potencial metastático desta subpopulação comparado com as demais células tumorais. Além disso, quando as células tumorais se desprendem do local primário do tumor e entram na circulação periférica são chamadas de células tumorais circulantes (CTCs), capazes de colonizarem linfonodos e outros órgãos, onde podem proliferar e originar eventuais tumores metastáticos. Nesse contexto, o presente trabalho inclui tanto uma abordagem experimental quanto uma revisão sistemática de literatura. A abordagem experimental teve como objetivo quantificar as CSC em tecido tumoral fresco proveniente de lesões primárias de CEB, buscando-se avaliar a associação das propriedades biológicas relacionadas ao fenótipo tronco tumoral e de TEM com o comportamento invasivo e metastático do CEB. Para esse fim, os níveis de expressão de CD44 e ESA foram avaliados por meio de citometria de fluxo em amostras teciduais frescas buscando-se identificar a subpopulação de CSC bem como suas frações $CD44^{+}ESA^{-}$ (em TEM/mesenquimal) e $CD44^{+}ESA^{+}$ (epitelial), sendo todos esses dados posteriormente relacionados com parâmetros clinicopatológicos. Os resultados revelaram que a subpopulação $CD44^{+}ESA^{-}$ (mesenquimal) foi significativamente associada ao grau de diferenciação tumoral e consumo de álcool, enquanto a subpopulação $CD44^{+}ESA^{+}$ (epitelial) foi diretamente correlacionada com a presença de invasão perineural e comprometimento de linfonodos regionais. De forma complementar, considerando-se a relevância das CTCs como biomarcadores minimamente invasivos da progressão do câncer, assim como sua plasticidade fenotípica, foi realizada uma revisão sistemática sobre as metodologias de detecção de CTCs especificamente em OSCC.

Palavras-chave: Carcinoma epidermóide de boca, células-tronco de câncer, transição epitélio-mesenquimal, plasticidade fenotípica, células tumorais circulantes, citometria de fluxo.

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1 Introduction

The oral squamous cell carcinoma (OSCC) is one of the most common malignant neoplasms of the head and neck region and still results in significant morbidity and mortality annually. The main regions affected include the mucosal epithelium of the oral cavity, including the tongue, floor of the mouth, buccal mucosa, alveolar ridges, retromolar trigone, and palate. The prognostic factor with the most significant impact on this disease is still the presence of metastasis in cervical lymph nodes, which occurs in 25 to 65% of cases (BAILLIE; ITINTEANG; YU; BRASCH *et al.*, 2016; BAUM; SETTLEMAN; QUINLAN, 2008; MARUR; FORASTIERE, 2016; SAVAGNER; KUSEWITT; CARVER; MAGNINO *et al.*, 2005; TURLEY; VEISEH; RADISKY; BISSELL, 2008; YOSHIDA; SAYA, 2016). Therefore, the capacity to predict tumor invasiveness and lymph node metastasis based on molecular parameters can positively influence treatment decisions and clinical outcomes (BAILLIE; ITINTEANG; YU; BRASCH *et al.*, 2016; MALIK; ZARINA; PENNINGTON, 2016; PETERSEN, 2009; WEINBERGER; MERKLEY; LEE; ADAM *et al.*, 2009).

The mechanisms of tumor recurrence and metastasis and treatment failure are due, among other factors, to the patterns of cell heterogeneity present in tumors. Two different models try to explain them. In the stochastic model, all cells that make up the tumor have indefinite proliferation and initiate new tumors. On the other hand, the cancer stem cell (CSC) hypothesis suggests that only this specific tumor cell subpopulation can extensively proliferate and start a new tumor growth (REYA; MORRISON; CLARKE; WEISSMAN, 2001). The definitive evidence for the existence of CSC in a specific type of tumor is the ability of only some tumor cells to initiate the disease in immunocompromised mice (BONNET; DICK, 1997; DE ANDRADE; RODRIGUES; RODINI; NUNES, 2017). CSC, also called tumor-initiating or tumorigenic cells, must likewise be able to reconstitute a heterogeneity of the human tumor from which they originated, including non-tumorigenic cells (RODINI; LOPES; LARA; MACKENZIE, 2017).

Given the importance of the presence of lymph node metastases and CSC in the clinical prognosis of cancer patients, recent studies have compared the expression and distribution of representative markers of CSC in primary tumors of different origins and corresponding metastatic lymph nodes in regards to the therapeutic response,

invasiveness, and prognosis. CD44 is a cell surface glycoprotein identified as a marker of CSCs in many solid tumors, and its role in tumor growth, metastasis spread, and resistance to therapies have been evidenced (EMICH; CHAPIREAU; HUTCHISON; MACKENZIE, 2015). In HNSCC, there is a direct correlation between CD44 expression, CSCs, and aggressiveness (ZHANG; FILHO; NÖR, 2012). Positive expression of CD44 marker has already been investigated and correlated with clinical parameters in OSCC, indicating a higher frequency of $CD44^{+}$ cells in patients with local tumor recurrence (ZEGERS-HOCHSCHILD; ADAMSON; DE MOUZON; ISHIHARA *et al.*, 2009). Although, there are few studies on the differential and comparative expression of markers of CSC in primary OSCC lesions and their possible clinical significance. *In vitro*, Fujinaga et al. (2014) investigated the differential expression of metastasis-related candidate genes in two OSCC tumor cell lines, one parental (WK2) and one metastatic derived from compromised cervical lymph nodes from the same patient (WK3F). The metastatic cell line showed significantly greater expression of the *SNAIL1* transcript and greater capacity for cell proliferation, migration, and invasion (FUJINAGA; KUMAMARU; SUGIURA; KOBAYASHI *et al.*, 2014). In the same context, our research group investigated whether the metastatic behavior of the SCC-9-LN1 cell line of OSCC could reflect its more significant commitment to the stem phenotype. Compared to the parental SCC-9, the metastatic cell line showed significantly higher expression of CD44 and BMI1 transcripts, in addition to the greater volumes of holoclones and tumorspheres formed, and the greater rates of migration and proliferation (LOPES, 2016).

The complex mechanism by which tumor cells leave the primary tumor, invade local tissues, and proceed to the development of metastases at distant sites is still unknown. However, it is proposed that CSC undergo an epithelial-mesenchymal transition (EMT) during this process. This biological program orchestrates intracellular changes such as upregulation of vimentin expression, inhibition of E-cadherin expression, and nuclear translocation of β-catenin, modifying the morphology and behavior of cells (GEWEILER; INHESTERN; BERNDT; GUNTINAS-LICHIUS, 2016). Thus, during EMT, CSC loses polarity and intercellular junctions, acquiring a migratory mesenchymal phenotype that enables them to migrate beyond the primary tumor and colonize distant sites, where they undergo the inverse mesenchymal-epithelial transition (MET) process to establish the

tumor of the same epithelial origin as the primary tumor (MOUSTAKAS; HELDIN, 2007). Biddle et al. were able to isolate four subpopulations of CSCs (Epi-S, Epi-P, pEMT-P, and pEMT-S) based on the profiles of CD44, CD24 and EpCAM (ESA) markers. The authors showed that the increase in phenotypic plasticity, represented by the ability to undergo the epithelial-mesenchymal transition (EMT) and reversion through the mesenchymal-epithelial transition (TEM), underlies the therapeutic resistance of CSC both in the epithelial phenotype ($CD44^{+}EpCAM^{high}CD24^{+}$) and in the post-EMT ($CD44^{high}EpCAM^{low/-}CD24^{+}$) phenotype (BIDDLE; GAMMON; LIANG; COSTEA *et al.*, 2016).

Second primary tumors and distant metastases can occur in up to 20% of head and neck cancer cases (FUCHS; FUJII; DORFMAN; GOODWIN *et al.*, 2008). Biologically, tumor metastasis occurs when cells from the primary tumor become invasive, targeting the surrounding lymphatic and blood vessels. When cells detach from the primary tumor site and enter the peripheral circulation, they are called circulating tumor cells (CTCs), capable of depositing in lymph nodes and other organs, where they can proliferate and give rise to possible metastatic tumors. These cells on circulation indicate that the disease has progressed to a stage susceptible to metastases (TSUKITA; FURUSE; ITOH, 2001).

The clinical relevance of detecting tumor cells disseminated via peripheral blood has already been demonstrated in several studies using samples from patients with solid epithelial tumors, including breast, lung, prostate, and colorectal (CHANG; WRIGHT; SVOBODA, 2007; GORDÓN-NUÑEZ; LOPRES; CAVALCANTE; HALBLEIB; NELSON, 2006; MAEDA; JOHNSON; WHEELOCK, 2005; PETRUZZELLI; TAKAMI; HUMES, 1999). Some studies have identified circulating tumor cells in the blood of patients with HNSCC, reporting that the presence and a more significant quantity of these cells were associated with a worse prognosis, indicating a high risk of local and distant recurrences and lower survival (RADISKY; LABARGE, 2008; TSUKITA; FURUSE; ITOH, 2001). Wang et al. (2012) identified both CTCs and CSCs circulating in breast cancer, analyzing their clinical relevance. In fact, these authors confirmed the existence of circulating CSCs, and detected statistical differences between positive and negative groups for this subpopulation and different TNM stages so that the presence and quantity of circulating CSCs correlated positively with regional lymph node metastasis (WANG; LIU; HUANG;

MA *et al.*, 2012). The presence of these circulating CSCs would then correspond to a risk, which could be quantified, of tumor dissemination and subsequent metastatic disease. The possibility of isolation and cultivation of these cells also favors the study of their biological behavior as well as their sensitivity to different therapies that can be implemented in the treatment of OSCC.

Although it is plausible to assume that the EMT phenomenon occurs in CSC present in the primary lesion of patients with OSCC, which end up reaching the bloodstream as CTCs, few studies in the literature proposed to detect CSC circulating in the peripheral blood of OSCC patients. CTCs reliable detection and characterization were possible only after a great improvement in technologic platforms. As CTCs are rare in the bloodstream, a sample enrichment protocol must precede the analysis based on their physical (by size, density, deformability, or surface charge) and biological (using specific protein biomarkers) features (YAP; LORENTE; OMLIN; OLMOS *et al.*, 2014; ZHANG; LIN; HUANG; WANG *et al.*, 2021). After isolation, analysis is usually done by immunostaining methodologies and RT-PCR techniques, depending on the aim of each study (YAP; LORENTE; OMLIN; OLMOS *et al.*, 2014).

CTCs have been explored in different cancers to early diagnosis, monitor therapies, and predict metastasis (ALIX-PANABIÈRES; PANTEL, 2013; LIANIDOU; STRATI; MARKOU, 2014). In breast, lung, and prostate cancer, high CTC counting was correlated with poor prognosis at various stages (VASSEUR; KIAVUE; BIDARD; PIERGA *et al.*, 2021). Similarly, many studies have also been conducted in HNSCC to establish the practical utility of CTC detection in the clinical setting and its potential role in the treatment and metastasis monitoring. However, a gold standard technique has not yet been defined for oral squamous cell carcinoma (OSCC), despite the wide range of methods used to detect and analyze these cells.

Our laboratory has investigated the role of CSC and microenvironment on EMT, invasion and metastasis in OSCC, using several *in vitro* and *in vivo* approaches, as well as patient-derived paraffin-embedded and fresh tissues. The aim of the present study was to quantify and characterize CSC subpopulations in fresh OSCC samples, evaluating the frequency of the CSC population ($CD44^+$) and its mesenchymal ($CD44^+ESA^-$) and

epithelial ($CD44^+ESA^+$) phenotypes with clinicopathological characteristics. In addition, a systematic literature review was performed on CTC detection methodologies in OSCC to serve as a basis for further studies investigating the CSC and EMT profiles found in CTCs.

2 Articles

The articles presented in this Thesis were written according to the Oral Oncology journal instructions and guidelines for article submission.

2.1 Article 1 – **Detection methodologies of circulating tumor cells in oral squamous cell carcinoma: a systematic review** (submitted).

2.2 Article 2 – **Frequency of cancer stem cells plastic subpopulations in oral squamous cell carcinoma fresh tumor specimens** (in preparation).

Article 1

Detection methodologies of circulating tumor cells in oral squamous cell carcinoma: a systematic review

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ABSTRACT

In recent decades, circulating tumor cells (CTCs) in solid tumors have been widely explored, and the results are promising. Although robust studies have evaluated CTCs in head and neck squamous cell carcinoma (HNSCC), there is still a lot to explore about these cells in oral squamous cell carcinoma (OSCC) specifically. This study aimed to evaluate the methodologies used to detect CTCs in the peripheral blood of patients with OSCC. Twenty-two eligible studies date from between 1999 and 2020, including 351 OSCC patients and more than 750 OSCC samples were analyzed. The results highlight the plethora of methodologies used for enrichment, isolation, and identification of CTCs in OSCC patients. However, there is no consensus on the most effective markers, and little is explored about the different possible phenotypes of these cells, which implies failures in approaches. The heterogeneity and plasticity of CTCs should be further investigated to define reliable markers for identifying cells with real metastatic potential and resistance to therapies.

Keywords: Circulating tumor cells, oral squamous cell carcinoma, peripheral blood, epithelial-mesenchymal transition, cancer stem cells.

BACKGROUND

In the last decades, there has been an intense effort to improve cancer diagnosis for greater efficiency, speed, and precision of the results. The development of non-invasive methods to diagnose and monitor tumor growth and treatment response is a significant challenge in oncology [1]. The major drawbacks of tissue biopsies from the primary tumor include the risk of complications due to the invasive nature of the procedure, lack of accessibility of some tumors, and a biased acquisition of sample due to intratumoral heterogeneity, that reflect only the molecular composition at the time of tissue resection [2, 3]. As tumors tend to be heterogeneous, possibly, some of their biological characteristics and the identification of their most plastic and invasive subclones can remain undetected, indicating that valuable information regarding tumor behavior might be missed [4]. Also, although tissue biopsies can deliver helpful information about the primary tumor genetic profile, there are limitations to its use as a method for monitoring post-treatment disease [3].

One of the most promising developments in cancer medicine has involved the discovery of CTCs as a minimally invasive biomarker [5]. CTCs are tumor cells that detach from solid tumors and spread via the circulatory system [6]. Although their existence has been known for decades, CTCs reliable detection and characterization were possible only after a great improvement in technologic platforms. As CTCs are rare in the bloodstream, a sample enrichment protocol must precede the analysis based on their physical (by size, density, deformability, or surface charge) and biological (using specific protein biomarkers) features [5, 7]. After isolation, analysis is usually done by immunostaining methodologies and RT-PCR techniques, depending on the aim of each study [5].

CTCs have been explored in different cancers to early diagnosis, monitor therapies, and predict metastasis [8, 9]. In breast, lung, and prostate cancer, high CTC counting was correlated with poor prognosis at various stages [10]. Similarly, many studies have also been conducted in HNSCC to establish the practical utility of CTC detection in the clinical setting and its potential role in the treatment and metastasis monitoring. However, a gold standard technique has not yet been defined to oral squamous cell carcinoma (OSCC), despite the wide range of methodologies used to detect and analyze these cells. Thus, this systematic review provides an overview of the CTCs detection methodologies applicable for OSCC patients.

METHODS

This qualitative systematic review was registered in the International Prospective Register of Systematic Reviews (PROSPERO; CRD42021245803). The protocol was written according to PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analyses) guidelines [11, 12].

Search strategy and study selection

An electronic database search was performed in the advanced mode of the PubMed® and Web of Science™ up to September 2021. The search strategy was customized according to the requirements of each database with the combination of keywords or terms as following: "oral squamous cell carcinoma", "oral cancer", "oral tongue squamous cell carcinoma", "oral cavity squamous cell carcinoma", "circulating tumor cells",

"circulating tumour cells", and "circulating neoplastic cells" (Supplementary information 1). A manual search in specific journals was carried out, and OpenGrey database was used to search the grey literature. All references were managed, and duplicates were removed using reference manager software (EndNote, Clarivate™). Article identification, screening and eligibility, and inclusion assessments were completed by one reviewer (NML) and confirmed by another one (RCO). Of note, only the information corresponding to CTCs in patients spacifically with OSCC was collected, even when the sample group was from HNSCC. The search strategy flowchart is detailed in **Figure 1**.

Eligibility criteria

The acronym PECO (Population/context, Exposure, Control, and Outcomes) was used to formulate the focused question of this systematic review: "What are the current methodologies for detecting CTC in OSCC?". The population was composed of blood samples from OSCC patients (P); CTCs identification (E); the comparison/control was blood samples from healthy donors when included (C); the rate of CTC detection (O).

Eligible studies included original research articles published in peer-reviewed journals, mainly focusing on human-based clinical trials, cross-sectional studies, prospective studies, cohort, and retrospective studies. The inclusion criteria were: 1) original texts published in a MEDLINE indexed journal; 2) only research techniques using peripheral blood; bone marrow, saliva, or other body fluids were not considered eligible; 3) studies conducted on patients with histologically confirmed OSCC, or HNSCC that discriminate results in samples from OSCC patients. Exclusion criteria were studies conducted as reviews, letters, editorials, case reports, case series, thesis, interviews, conference papers, and mainly studies that did not detail the technical conduction of CTC analyses or were written in languages other than English.

Risk of bias (RoB)

Two reviewers (NML and RBN) independently assessed the methodological quality of the selected studies according to their level of evidence, as proposed by the Joanna Briggs Institute Methodological Index [13, 14], with some adjustments, according to the study design proposed in each article selected. Doubts and discrepancies between the

investigators were discussed to enter a consensus. In the final analysis, studies were categorized as "high RoB (Risk of Bias)" when the study achieved up to a 49% "yes" score; "Moderate RoB" when the study reached 50% to 69% "yes" score; and "low RoB" when the study reached over 70% "yes" score.

Data collection and analysis

The data were collected from the papers selected by the same reviewers (NML and RCO). The extracted data were summarized through the narrative with an overview of the year and the country of publication, general purpose of the study, number of OSCC patients and their disease staging, number of controls and the use of spiking assays, methodologies of CTC enrichment and detection, data of collection time and type of treatment conducted, and finally, the cutoff value for CTC detection and positive rate. Of note, only the information corresponding to CTCs in patients with OSCC was collected.

RESULTS

Search results

Using the search strategy reported, 599 studies were retrieved initially. A manual search included 13 additional studies in the present review from a thorough search for the references of the selected articles. After analyzing 612 titles, 527 studies were excluded. Reading the abstracts of the remaining 85 articles, 41 were further selected for a full reading. Nineteen studies were excluded because they did not show the necessary data for the extraction (**Supplementary information 2**). Thus, twenty-two complete texts had satisfactory data captured and were included in this systematic review (**Figure 1**).

Study characteristics

The eligible 22 studies were published from 1999 to 2020, and they included 351 patients diagnosed with OSCC, a total of more than 750 samples analyzed. Seventeen studies (77.27%) evaluated samples from patients histologically diagnosed with HNSCC, including lesions in different sites. However, in these studies, only data from OSCC patients were extracted. Although 7 studies (31.82%) did not discriminate the stage of the disease of OSCC patients, and 8 others (36.36%) included all stages (I - IV); four studies

(18.18%) included samples from stages II to IV, and 3 (13.64%) focused on locally advanced-stage of disease (III - IV). Of those last mentioned, Brakenhoff et al. [15] and Wang et al. [16] also included OSCC patients with local recurrence, and Chikamatsu et al. [17] included only patients with recurrent tumor and/or distant metastasis (4.55%).

Blood sample collection was performed before any treatment in 18 studies (81.82%), and 9 of them (40.91%) also included samples collected during and/or after treatment. Most studies (81.82%) used peripheral blood from healthy volunteers as controls to ensure efficient CTC detection, and 13 studies (59.09%) reported having performed tumor cell spiking assays. The cutoff value varied according to each study. Samples were considered positive when at least 1 CTC was detected in 1 to 30 mL of peripheral blood. Differently, Weller et al. [18] considered positive cases with at least 1 CTC for every 1000 mononuclear cells in 20 mL of peripheral blood.

The data reported in the selected literature showed diverse methodologies for enrichment and detection of CTC in OSCC. Lysis of erythrocytes, centrifugation, or sedimentation was used by at least 15 studies (68.18%) for tumor cell enrichment. PBMC was used as starting material for the analyses in 15 (68.18%) of the studies, and 4 of them used a density media gradient to improve the buffy coat isolation. Six studies (27.27%) used negative selection based on depletion of CD45 positive cells, while all articles performed positive selection using at least one epithelial marker. Among the most frequent markers, cytokeratin (CK) was used by 18 studies (81.82%), followed by EpCAM in 11 (50%), and EGFR in 8 (36.36%). Combined markers were used in 11 studies (50%), considering CTCs as CD45 negative and epithelial marker positive, as idealized in the commercial CellSearch® system used by 3 of them. Immunostaining techniques were widely used (54.55%) with the same frequency as polymerase chain reaction (PCR) technics (54.55%). Size-based CTC detection technique was used for Kawada et al. [19] and Tada et al. [20] using filtration systems containing pores with 7 µm diameter (9.09%). The complete data extraction of the studies, including positivity rates for CTC detection, were summarized in **Table 1**.

The risk of bias assessment was evaluated according to Joana Briggs Institute (JBI) criteria [13, 14]. Among the included papers, fifteen (68.18%) studies showed a good-

quality and low risk of bias, three (13.63%) presented a moderate risk of bias, whereas four (18.18%) did not fulfill the requirements according to the JBI, presenting a high risk of bias (**Supplementary information 3**). Overall, the main biases identified were 1) did not provide sufficient information about diagnosis criteria, 2) did not assess the presence of confounding factors and/or did not report a strategy to deal with them, 3) unclear answers regarding the post-intervention clinical condition.

DISCUSSION

Circulating tumor cells in oral squamous cells carcinoma

HNSCC is a broad and heterogeneous group of malignant tumors affecting different anatomical sites at the head and neck. Specifically, OSCC arises from the mucosal epithelium of the oral cavity, including the tongue, floor of the mouth, buccal mucosa, alveolar ridges, retromolar trigone, and palate. The main risk factors for oral cavity carcinogenesis include tobacco exposure and alcohol consumption, as well as human papillomavirus (HPV) persistent infection in some cases [37, 38]. Although systematic reviews about CTCs in HNSCC have been published, little is addressed about the incidence of these cells in OSCC. In 2014, Wikner et al. proposed summarizing the technologies currently used to detect CTCs in OSCC; nevertheless, most articles selected (80%) reported HNSCC data in general [39]. Since OSCC has its own development, complexity, and molecular pathways [40], the present review includes HNSCC studies but carefully analyzed exclusively the results of OSCC patients, showing a scenario of methodologies and specific results for CTCs of the oral cavity origin.

Undoubtedly, the prognostic factor with most significant impact in OSCC patients is metastasis, mainly in cervical lymph nodes, detected at the time of diagnosis in 50% of cases [41, 42]. Biologically, metastasis development starts when some primary tumor cells become invasive and direct towards surrounding lymphatic/blood vessels, reach lymph nodes and other tissues, where they can proliferate and originate new tumor mass [43]. Thus, the presence of CTCs in the circulation indicates that the disease has progressed to a stage where metastasis is possible [43-45]. In the systematic review of Wu et al., the presence of CTC indicated a worse disease-free survival for HNSCC

patients, whatever the biological approaches used [46]. Another study by Wang et al., reported that recurrence and/or metastasis rate was significantly higher in the CTC-positive group, indicating the predictive value of the detection of CTCs in patients with HNSCC, especially on the tumor progression [47].

Quality of eligible studies

This systematic review was based on 22 studies, most of which (81.81%) presented good quality according to JBI criteria, indicating a low or moderate risk of bias.

Considering that the focus of this review is on the methodology conducted in the studies, it is essential to assess the number of samples analyzed and the controls included. The number of samples (n) varied considerably between the studies, due in part to the restriction on using data relating to the OSCC: even though the study includes a large sample of HNSCC, only the percentage referring to the oral cavity was extracted. Negative controls based on blood samples from healthy volunteers were widely used in selected studies and provided reliability to the results. Only five articles (22.72%) did not use or did not specify their use.

Spiking experiments are usually performed to confirm the effectiveness of detection methodologies. In these, a known number of cells from a cultured tumor cell lineage are mixed with peripheral blood samples from healthy volunteers, which go through the same steps as the samples studied. In order to validate CTC investigation methodology, tumor cells must be efficiently detected in this scenario. Of the 13 studies that performed this assay, only four (30.76%) used it as a positive control, considering the presence/absence of CTCs. In comparison, nine studies (69.23%) aimed to measure assay sensitivity or detection limit using known cell concentrations, determining the assertiveness level of their methodologies. HNSCC cell lines were used in most studies, ensuring better representation of the tumor cells sought. Only two studies used colon adenocarcinoma cell lines SW480 [22] and SW620 [31], which, despite also having an epithelial origin, may not share the same molecular profile as oral tumor cells. In contrast, Weller et al. reported using different cell lines, including epithelial, mesenchymal, and stem-like cells (CD133+), ensuring the detection of multiple cell profiles within tumor heterogeneity [18].

Circulating tumor cells detection methodologies

Detecting CTCs remains technically challenging, especially in OSCC patients. CTCs occur at very low concentrations; for example, a single tumor cell in a background of millions of blood cells [8]. Generally, the procedure for identifying CTCs comprises two steps based on their physical and chemical properties. In the first step, enrichment techniques are necessary to sort the extremely rare CTCs from peripheral blood cells. The most common separation mechanisms are immunomagnetic assays, microfluidic chips, size-selective isolation, density gradient centrifugation, dielectrophoretic field forces methodologies, and in vivo approaches [5, 48]. Subsequently, the identification and analysis of CTCs are performed using genetic, protein, and functional assays [7, 49]. Most of the selected studies used density-based enrichment methods such as centrifugation or sedimentation. Despite having low sensitivity [49], it is a simple, cheap, and accessible alternative, which does not involve the choice of markers. From 1999 to 2008, five studies included in this review exclusively used this type of enrichment combined with lysis of erythrocytes before RT-PCR analysis [15, 21, 22, 26, 28].

Targeting epithelial antigens was the first approach to isolate CTCs among all blood cells of patients with cancers of epithelial origin [50]. The negative selection based on depletion of CD45 (common leukocyte antigen) positive cells combined with the positive selection using epithelial markers has been used widely for a long time. Despite the most frequent use of a specific CK or a set (pan-CK) to detect CTCs, positive selection by EpCAM was one of the first techniques used to detect CTC in the whole blood of OSCC patients. The best-known example of this methodology is the CellSearch® technology, the only technique for enumerating CTCs from patients in the clinic cleared by the Food and Drug Administration agency (FDA/USA) for metastatic breast, prostate, and colorectal cancer [51]. The technique, used by three selected studies [32, 33, 35], defines a CTC according to positivity for EpCAM and Pan-CK, and negativity of CD45 expression, using a IMS and IFF imaging technology. However, the potentially more metastatic CTCs remain undetected by EpCAM-based enrichment methods, due to its low/absence expression during the epithelial-mesenchymal transition (EMT) state. In many cancers, this mesenchymal-like subpopulation of cells was correlated with poor outcomes, indicating

that this phenotypic change is advantageous in circulation and other sites [52, 53]. Tada et al. used four representative epithelial markers for CTC detection in HNSCC blood samples. Among the 44 samples tested, EpCAM was detected in 13.6%, EGFR in 15.9%, c-Met in 27.3%, and KRT19 in 47.7%. Only three patients were positive for all four markers, 1 for three markers, 7 for two markers, and 17 for one marker [20]. These data suggest that an epithelial marker that can be used solely to isolate all possible phenotypes of CTCs, including mesenchymal CTCs undergoing EMT, is still unknown.

Epithelial-mesenchymal transition, cancer stem cells, and circulating cancer stem cells

Metastasis establishment is complex and includes several biological processes, including the EMT, invading the circulating blood and reverting back through mesenchymal-epithelial transition (MET) to colonize other tissues [54, 55]. This sequence of events configures high cellular plasticity, and instability of surface markers is generally used to identify the origin of these cells. Therefore, the use of enrichment and detection methodologies that do not depend on the expression of surface epithelial markers has been encouraged to avoid failure to detect CTCs undergoing EMT, including the mesenchymal-like subpopulations [48, 56]. Considering this scenario, two articles selected in this systematic review used enrichment methodologies independent of surface epithelial markers to detect CTCs in OSCC. Weller et al. used density enrichment and detected CTCs with epithelial properties (CK+/Ncad-/CD45- and CK+/CD133-/CD45-) as well as CTCs with mesenchymal features (N-cad+/CK-/CD45-) and CTCs with both phenotypic characteristics (N-cad+/CK+/CD45-) by immunofluorescence [18]. Balasubramanian et al. used centrifugation and immunomagnetic negative selection enrichment based on CD45 marker expression. They evaluated two distinct profiles of CTCs with mesenchymal markers expression by immunofluorescence; one positive for an epithelial marker (CK+/Vimentin+/EGFR, CD44, or N-cad+) and another one negative (CK-/Vimentin+/EGFR, CD44, or N-cad+), suggesting that the CTCs have undergone the EMT process [34]. Furthermore, positivity for CD44, a strong cancer stem cells (CSC) marker, suggests that these EMT cells may be circulating CSCs.

Given the fact that to establish a new tumor in a distant location, malignant cells need to survive the unfavorable environment of the bloodstream, evade the immune response, and extravasate at a distant location, it is reasonable to consider that only circulating CSCs would have that ability [54]. Patel et al. isolated CD44+ cells from OSCC peripheral blood samples using immunomagnetic cell separation and validated stem-like CTCs using flow cytometry techniques. These CD44+CD24-CD45- cells demonstrated increased sphere-forming capability and intrinsic chemo-resistance compared to CD44- cells [57]. Tada et al. evaluated the expression of CSC markers (CD44, NANOG, and ALDH1A1) and key EMT markers (SNAI1 and VIM) in HNSCC. In 28 CTC-positive samples, they reported heterogeneous expression of the five markers, including positive samples for all and samples that did not express any level of them [20]. These data support the hypothesis of the presence of CSCs in the circulation and likewise show the phenotypic variation of these cells during EMT.

Circulating tumor cells in the course of OSCC treatment

It is known that the morphology of CTCs varies depending on the disease, time until diagnosis, and treatment timing [58]. Thus, as important as knowing the phenotype of CTCs that initiate metastases is to understand at what point in the disease these cells are released into the bloodstream. OSCC is generally treated with surgical resection, followed by adjuvant chemoradiotherapy depending on the disease stage [37]. Nine articles selected in this systematic review evaluated peripheral blood samples collected at different times of treatment. Constantly comparing with data collected before any interference (preoperative samples), some studies analyzed samples during treatment with radiotherapy and/or after therapy and surgical resection. However, the wide range of methodologies and number of participants applied hinders the comparison of the results. It is worth mentioning the study of Partridge et al., which collected blood samples preoperatively, intraoperatively (after the resection was complete), and three months post-surgery (once radiotherapy was complete), and used negative CD45 based enrichment, non-based EpCAM methodology, and approaches incorporating ICC for Pan-CK, and RT-PCR for E48 (also known as Ly-6D, lymphocyte antigen 6 family member D). The authors found that more than a third of the pre and intraoperative samples were positive for the

evaluated markers. However, there was no detection in the postoperative samples, suggesting that cancer cells overflow into the circulation when the tumor mass is high [25].

Two studies stand out for analyzing samples collected before and immediately after incisional biopsies or surgical resection. The authors suggest that cell clusters are released into the bloodstream during the malignant oral epithelial lesion interference [15, 26, 28]. This approach must be interpreted with caution because cells that enter the bloodstream by mechanical action are probably not cells with plastic potential; therefore, they do not present the capacity to generate metastases.

Association with clinical features

In this systematic review, most studies (77.27%) aimed to correlate the presence of CTCs with clinicopathological data of patients. However, little could be considered about statistical correlations between the presence of CTCs and clinicopathological data from patients specifically with OSCC. Balasubramanian et al. updated Kaplan-Meier disease-free survival plot from the publication by Jatana et al., concerning the number of CTCs per mL of HNSCC patient blood at the time of surgery, demonstrating the potential of CTCs as a prognostic marker of disease-free survival in OSCC [30, 34]. Significant positive correlations could be found by Grobe et al. for CTC detection, advanced tumor sizes, and distant metastasis. The presence of CTCs was also found to be the strongest independent predictor of locoregional tumor relapse [35].

Although some selected HNSCC studies have performed correlation analyses between CTCs and clinicopathological data, statistical results specific to OSCC patients have not been reported. Other studies have included such a low sample size of oral cavity HNSCC sources that such correlations would be impractical.

Prospective studies involving large health centers with considerable OSCC patients are needed to allow strong clinicopathological and prognostic data correlations. Prospective studies with standardized definitions of CTCs, including the most clinically relevant method of identification, are thus urgently needed to exploit the full potential of CTCs as prognostic, predictive, and intermediate endpoint markers [5]. However, there is

still a long way to explore the multiple phenotypes of CTCs for the most effective detection methods to emerge.

Future perspectives

An accurate capture and identification methodology must be established to detect CTCs in OSCC patients, considering the different possible cellular profiles in the disease progression. It is essential to know the phenotypic profile of these cells and understand their biology to identify them effectively. Efficient molecular markers have to be elected to improve the detection of CTCs in OSCC progression, including targets to select circulating CTCs undergoing EMT as well as investigating their CSC phenotype.

While the use of CTCs for OSCC diagnosis has not yet been reported, the great immediate potential of CTC detection is certainly disease monitoring [59]. Considering routine clinical use, systems that provide greater precision and yield will be the most convenient, together with the flexibility of isolation of different phenotypes of CTCs [48]. A blood sample collection to investigate CTCs could be part of the OSCC patient follow-up after resection, ideally for periods longer than three years.

The quantification of CTCs in peripheral blood of OSCC patients will be a clinically valuable and feasible alternative. Future personalized medicine strategies for OSCC patients will be based on liquid biopsy, with subsequent and rapidly reported molecular analysis of CTCs. Although certainly promising, this scenario is technically challenging.

CONCLUSIONS

The wide variety of methodologies used in studies of CTCs in OSCC exposes the lack of a consensus about the phenotype of these cells. For research to advance, the heterogeneity and plasticity of CTCs must be considered, and reliable markers must be defined for identifying cells with real metastatic potential and resistance to therapies. Investigating the presence of CTCs during the entire follow-up of patients, from diagnosis to discharge, would provide valuable results for the future establishment of clinical approaches in the management of patients with OSCC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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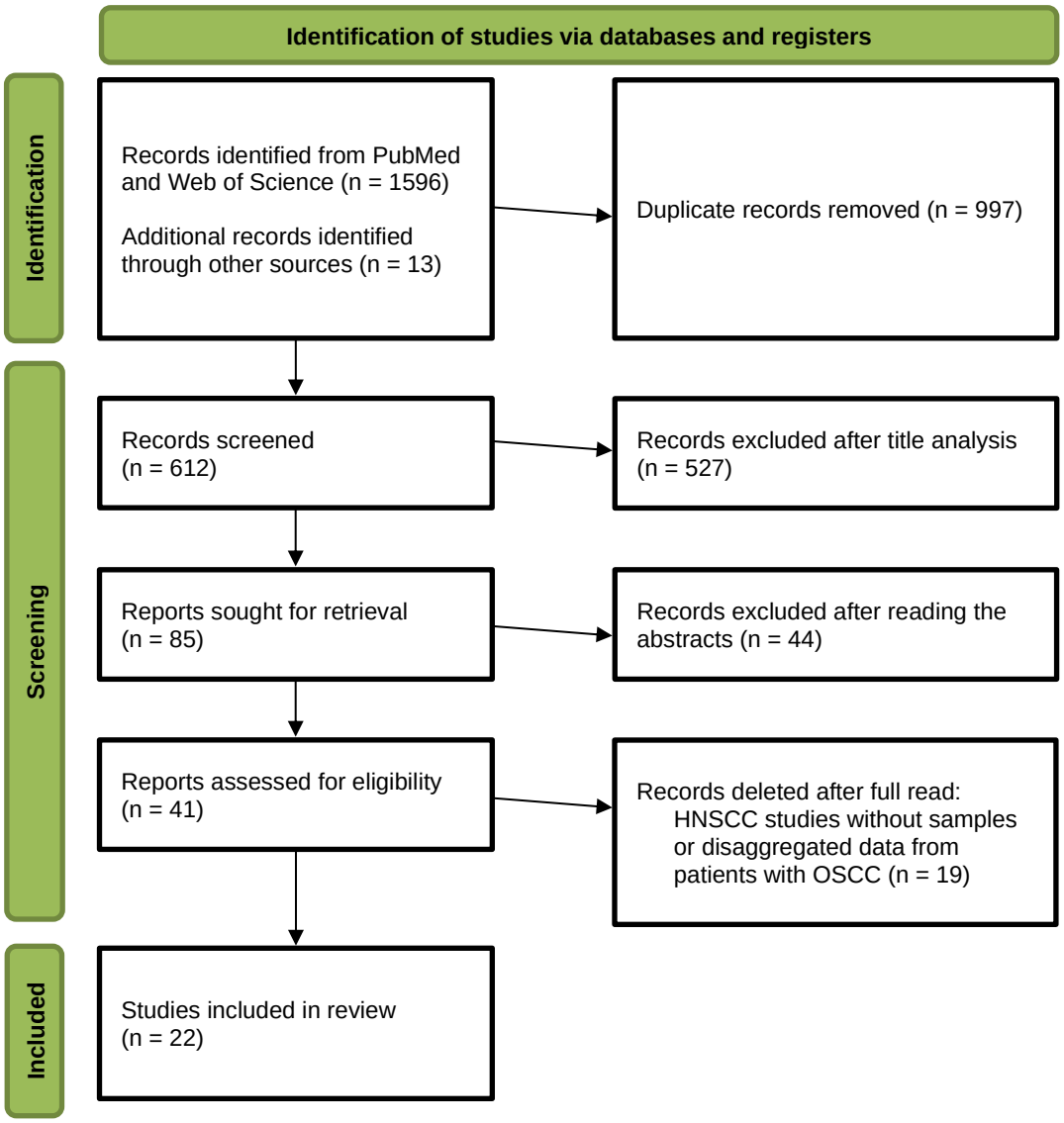

Figure 1 - Flow diagram of the strategy used for the selection of reports (according to PRISMA guidelines).

Table 1 - Baseline characteristics of the enrolled studies.

Ref.	First author (Country)	Year	General-purpose	OSCC stage	OSCC (n)	Samples + controls	Spiking assay	CTC enrichment and detection	Sample time	Treatment	Cutoff value*	Positive rate
[21]	Kawamata, H. (Japan)	1999	CTC detection and clinical data correlations	I - IV	12	12 + 17	Yes	Centrifugation, lysis of erythrocytes, RNA extraction, and RT-PCR (GAPDH, CK20)	Pre/Post	Surgery, radiotherapy, chemotherapy	5 mL	Pre: 11 (91.66%) Post: 0 (0%)
[15]	Brakenhoff, R. H. (Holland)	1999	Assay description for CTC detection in BM and PB	II - IV Recurrence	18	36 + 29	Yes	Centrifugation, lysis of erythrocytes, RNA extraction, and RT-PCR (E48)	Pre/Intra	Surgery	7 mL	Pre: 2 (11.11%) Intra: 0 (0%)
[22]	Pajonk, F. (Germany)	2001	CTC detection and clinical data correlations	NS	13	>39 + 12	Yes	Dextran sedimentation, lysis of erythrocytes, RNA extraction, and RT-PCR (β-actin, CK19)	Pre/Intra /Post	Radiotherapy	5 mL	Pre: 0 (0%) Intra: 0 (0%) Post: 1 (7.69%)
[23]	Wirtschafter, A. (USA)	2002	CTC detection and clinical data correlations	III - IV	2	2 + 0	No	PBMC isolation (density media gradient), positive IMS (EpCAM), and ICC (Pan-CK)	Pre	Surgery	30 mL	1 (50%)
[24]	Zen, H. (Japan)	2003	CTC detection, intravasation analysis, and survival in the circulation.	I - IV	21	21 + 8	Yes	RNA extraction, RT-PCR and RT-qPCR (GFP, GAPDH, SCCA, EGFR)	NS	NS	5 mL	RT-PCR SCCA: 1 (4.76%) EGFR: 2 (9.52%) RT-qPCR SCCA: 11 (52.38%) EGFR: 6 (28.57%)
[25]	Partridge, M. (United Kingdom)	2003	CTC detection in BM and PB and clinical data correlations	I - IV	39	117 + 50	Yes	Centrifugation, lysis of erythrocytes, RNA extraction, and RT-PCR (E48); Negative IMS (CD45) and ICC (Pan-CK)	Pre/Intra /Post	Surgery and radiotherapy	7 mL	Pre: 14 (35.89%) Intra: 14 (35.89%) Post: 0 (0%)
[26]	Ramani, P. (India)	2005	CTC dissemination from incisional biopsy	I - IV	10	20 + 5	No	Centrifugation, lysis of erythrocytes, RNA extraction, and RT-PCR (β-actin, CK19)	Pre/Post 15min	Incisional biopsies	5 mL	Pre: 0 (0%) Post: 0 (0%)
[27]	Guney, K. (Turkey)	2007	CTC detection and clinical data correlations	II - III	6	6 + 0	No	PBMC isolation (density media gradient) and positive IMS (EpCAM)	Pre	Surgery	30 mL	1 (16.66%)
[28]	Dyavanagoudar, S. (India)	2008	CTC dissemination from incisional biopsy and analysis of detection methodology	II - IV	25	75 + 10	No	Centrifugation, lysis of erythrocytes, RNA extraction and RT-PCR (CK19)	Pre/Post 15 and 30min	Incisional biopsies	1 mL	Pre: 0 (0%) Post 15min: 4 (16%) Post 30min: 0 (0%)
[29]	Winter, S. C. (Australia)	2009	CTC detection and clinical data correlations	I - IV	9	36 + NS	Yes	Positive IMS (EpCAM, MUC1) and RT-PCR (ESX, EGFR, EphB4, CK19)	Pre/Post	Surgery	10 mL	Pre: 7 (77.77%) Post: 7 (77.77%)
[30]	Jatana, K. R. (USA)	2010	CTC detection and clinical data correlations	NS	25	25 + 10	No	Lysis of erythrocytes, negative IMS (CD45) and ICC/IF (Pan-CK, DAPI)	Pre	Surgery	1 mL	19 (76 %)
[31]	Hristozova, T. (Germany)	2011	CTC detection and clinical data correlations	NS	9	9 + 30	Yes	Negative IMS (CD45) and flow cytometry (EpCAM, Pan-CK, CD45); Centrifugation, lysis of erythrocytes, RNA extraction, and Nested RT-PCR (EGFR)	Pre	Radiotherapy, chemotherapy	3.75 mL	6 (67%)

Table 1 - Baseline characteristics of the enrolled studies (continuation).

Ref.	First author	Year	General-purpose	OSCC stage	OSCC (n)	Samples§ + controls	Spiking assay	CTC enrichment and detection§	Sample time§	Treatment§	Cutoff value‡	Positive rate§
[16]	Wang, Xu (USA)	2011	CTC detection and clinical data correlations	I - IV Recurrent	11	11 +3	Yes	PBMC isolation (density media gradient), SERS (EGFR) and ICC (CK, EGFR)	NS	NS	1 mL	10 (90.90%)
[32]	Buglione, M. (Italy)	2012	CTC detection and clinical data correlations	NS	3	3 + 9	No	CellSearch® system (DAPI, EpCAM, CK, CD45)	Pre	NS	7.5 mL	0 (0%)
[33]	Nichols, A. C (Canada)	2012	CTC detection and clinical data correlations	III - IV	8	8 + 4	Yes	CellSearch® system (DAPI, EpCAM, CK, CD45)	Pre	Surgery, radiotherapy, chemotherapy	7.5 mL	2 (25%)
[34]	Balasubramanian, P. (USA)	2012	CTC detection, clinical data correlations, and analysis of the methodology	II - IV	8	11 + 10	No	Centrifugation, lysis of erythrocytes, negative IMS (CD45) and IF (DAPI, Pan-CK, EpCAM, VIM, EGFR, CD44, N-cad)	Pre and/or Post	Surgical resection	1 mL	CK+ Pre: 3 (100%) CK+ Post: 8 (100%) CK- Pre: 2 (66.66%) CK- Post: 6 (75%)
[18]	Weller, P. (Germany)	2014	Types of CTC and clinical data correlations	IV	1	2 + 12	Yes	PBMC isolation (density media gradient) and IF (DAPI, Pan-CK, N-cad, CD133, CD45)	Pre/Post	Curative resection	1000 PBMNC	Pre: 1 (100%) Post: 1 (100%)
[35]	Gröbe, A. (Germany)	2014	Presence of DTC in BM and CTC in PB, and clinical data correlations	I - IV	80	80 + 0	No	CellSearch® system (DAPI, EpCAM, CK, CD45)	Pre	Surgery	7.5 mL	10 (12.5%)
[36]	Tinhofer, I (Germany)	2014	CTC detection and clinical data correlations	I - IV	40	40 + 0	No	Centrifugation, lysis of erythrocytes, RNA extraction and Nested RT-PCR (EGFR)	Post	Surgery, radiotherapy, chemotherapy	7.5 mL	10 (25%)
[19]	Kawada, T. (Japan)	2017	CTC detection and clinical data correlations	NS	4	4 + 3	Yes	Low-pressure filtration system (size exclusion) and IF (DAPI, Pan-CK, CD45, EpCAM)	Pre	Surgery, radiotherapy, chemotherapy	7.5 mL	4 (100%)
[17]	Chikamatsu, K. (Japan)	2019	CTC detection and immune-regulatory molecules expression	Recurrent and/or metastatic	3	3 + 20	Yes	PBMC isolation (density media gradient), lysis of erythrocytes, negative IMS (CD45), and RT-qPCR (β-actin, CK19, EpCAM, EGFR, c-Met, PDL1, PDL2, CD47)	NS	NS	7.5 mL	1 (33.33%)
[20]	Tada, H. (Japan)	2020	CTC detection, characterization, and clinical data correlations	NS	4	4 + 1	Yes	CellSieve™ microfilter, ICC (Pan-CK, EpCAM, CD45), and RT-qPCR (β-actin, EPCAM, MET, KRT19, EGFR, PIK3CA, CCND1, SNAI1, VIM, CD44, NANOG, ALDH1A1, CD47, CD274, PDCD1LG2)	Pre	Surgery, radiotherapy, chemotherapy	7.5 mL	3 (75%)

Ref., reference; IMS, immunomagnetic separation; ICC, immunocytochemistry; IF, immunofluorescence; SERS, surface-enhanced Raman spectroscopy; PBMNC, peripheral blood mononuclear cells; Pre, preoperative; Intra, intraoperative; Post, postoperative; NS, not specified; CK, cytokeratin; EpCAM, epithelial cell adhesion molecule; EGFR, epidermal growth factor receptor; VIM; vimentin; SCCA, squamous cell carcinoma antigen; c-Met, mesenchymal-epithelial transition factor; ALDH1, aldehyde dehydrogenase 1; N-cad, N-cadherin; PB, peripheral blood; BM, bone marrow. §In HNSCC studies, these data were extracted exclusively from patients with oral cavity cancer. ‡Cutoff value: at least one CTC detected in the specified PB volume.

Supplementary information 1: Search strategy in the databases.

Database	Search
PubMed	**#1** “circulating tumor cells” OR “circulating tumour cells” OR “CTC” OR “CTCs” OR “circulating neoplastic cells” AND/OR **#2** “oral squamous cell carcinoma” OR “oral cancer” OR “oral tongue squamous cell carcinoma” OR “oral cavity squamous cell carcinoma” OR “squamous cell carcinoma of the mouth”
Web of Science	**#1** TS=(“circulating tumor cells” OR “circulating tumour cells” OR “CTC” OR “CTCs” OR “circulating neoplastic cells”) AND/OR **#2** TS=(“oral squamous cell carcinoma” OR “oral cancer” OR “oral tongue squamous cell carcinoma” OR “oral cavity squamous cell carcinoma” OR “squamous cell carcinoma of the mouth”)
Open Grey	**#1** “circulating tumor cells” OR “circulating tumour cells” OR “CTC” OR “CTCs” OR “circulating neoplastic cells” AND/OR **#2** “oral squamous cell carcinoma” OR “oral cancer” OR “oral tongue squamous cell carcinoma” OR “oral cavity squamous cell carcinoma” OR “squamous cell carcinoma of the mouth”

Supplementary information 2: Excluded articles after full-text review (chronological order).

Partridge M, Phillips E, Francis R, Li SR. Immunomagnetic separation for enrichment and sensitive detection of disseminated tumour cells in patients with head and neck SCC. J Pathol. 1999;189:368-77.

Yang L, Lang JC, Balasubramanian P, Jatana KR, Schuller D, Agrawal A, et al. Optimization of an enrichment process for circulating tumor cells from the blood of head and neck cancer patients through depletion of normal cells. Biotechnol Bioeng. 2009;102:521-34.

Balasubramanian P, Yang L, Lang JC, Jatana KR, Schuller D, Agrawal A, et al. Confocal images of circulating tumor cells obtained using a methodology and technology that removes normal cells. Mol Pharm. 2009;6:1402-8.

Hristozova T, Konschak R, Budach V, Tinhofer I. A simple multicolor flow cytometry protocol for detection and molecular characterization of circulating tumor cells in epithelial cancers. Cytometry A. 2012;81:489-95.

Tinhofer I, Hristozova T, Stromberger C, Keilhoiz U, Budach V. Monitoring of circulating tumor cells and their expression of EGFR/phospho-EGFR during combined radiotherapy regimens in locally advanced squamous cell carcinoma of the head and neck. Int J Radiat Oncol Biol Phys. 2012;83:e685-90.

Lin HC, Hsu HC, Hsieh CH, Wang HM, Huang CY, Wu MH, et al. A negative selection system PowerMag for effective leukocyte depletion and enhanced detection of EpCAM positive and negative circulating tumor cells. Clin Chim Acta. 2013;419:77-84.

He S, Li P, Long T, Zhang N, Fang J, Yu Z. Detection of circulating tumour cells with the CellSearch system in patients with advanced-stage head and neck cancer: preliminary results. J Laryngol Otol. 2013;127:788-93.

Bozec A, Ilie M, Dassonville O, Long E, Poissonnet G, Santini J, et al. Significance of circulating tumor cell detection using the CellSearch system in patients with locally advanced head and neck squamous cell carcinoma. Eur Arch Otorhinolaryngol. 2013;270:2745-9.

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Hsieh JC, Lin HC, Huang CY, Hsu HL, Wu TM, Lee CL, et al. Prognostic value of circulating tumor cells with podoplanin expression in patients with locally advanced or metastatic head and neck squamous cell carcinoma. Head Neck. 2015;37:1448-55.

Grisanti S, Almici C, Consoli F, Buglione M, Verardi R, Bolzoni-Villaret A, et al. Circulating tumor cells in patients with recurrent or metastatic head and neck carcinoma: prognostic and predictive significance. PLoS One. 2014;9:e103918.

Fanelli MF, Oliveira TB, Braun AC, Corassa M, Abdallah EA, Nicolau UR, et al. Evaluation of incidence, significance, and prognostic role of circulating tumor microemboli and transforming growth factor-β receptor I in head and neck cancer. Head Neck. 2017;39:2283-92.

Strati A, Koutsodontis G, Papaxoinis G, Angelidis I, Zavridou M, Economopoulou P, et al. Prognostic significance of PD-L1 expression on circulating tumor cells in patients with head and neck squamous cell carcinoma. Ann Oncol. 2017;28:1923-33.

Morgan TM, Wang X, Qian X, Switchenko JM, Nie S, Patel KR, et al. Measurement of circulating tumor cells in squamous cell carcinoma of the head and neck and patient outcomes. Clin Transl Oncol. 2019;21:342-7.

Onidani K, Shoji H, Kakizaki T, Yoshimoto S, Okaya S, Miura N, et al. Monitoring of cancer patients via next-generation sequencing of patient-derived circulating tumor cells and tumor DNA. Cancer Sci. 2019;110:2590-9.

Garrel R, Mazel M, Perriard F, Vinches M, Cayrefourcq L, Guigay J, et al. Circulating Tumor Cells as a Prognostic Factor in Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma: The CIRCUTEC Prospective Study. Clin Chem. 2019;65:1267-75.

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Tada H, Takahashi H, Kawabata-Iwakawa R, Nagata Y, Uchida M, Shino M, et al. Molecular phenotypes of circulating tumor cells and efficacy of nivolumab treatment in patients with head and neck squamous cell carcinoma. Sci Rep. 2020;10:21573.

Supplementary information 3: Risk of bias

Authors	Design	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	%	Risk
Kawamata et al., 1999	Case-control	Y	U	U	Y	U	Y	U	Y	Y	U	----	50,00	Moderate
Brakenhoff et al., 1999	Cohort	Y	U	U	U	N	Y	Y	N	U	U	U	27,30	High
Pajonk et al., 2001	Cohort	Y	Y	Y	U	U	Y	Y	Y	Y	Y	NA	80,00	Low
Wirtschafter et al., 2002	Cross-sectional	Y	Y	U	U	Y	U	Y	NA	----	----	----	57,14	Moderate
Zen et al., 2003	Cross-sectional	Y	Y	Y	Y	U	U	Y	Y	----	----	----	75,00	Low
Partridge et al., 2003	Cohort	Y	U	U	Y	Y	Y	Y	Y	Y	Y	Y	81,81	Low
Ramani et al., 2005	Case-control	Y	Y	Y	Y	U	U	U	Y	Y	U	----	60,00	Moderate
Guney et al., 2007	Cohort	Y	Y	U	Y	Y	Y	Y	Y	Y	NA	U	80,00	Low
Dyavanagoudar et al., 2008	Case-control	Y	U	Y	Y	Y	N	N	Y	Y	Y	-----	70,00	Low
Winter et al., 2009	Cohort	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100,00	Low
Jatana et al., 2009	Cohort	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	100,00	Low
Highistozova et al., 2011	Cohort	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	100,00	Low
Wang et al., 2011	Case-control	U	U	U	U	U	N	N	Y	Y	U	-----	10,00	High
Buglione et al., 2012	Cohort	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100,00	Low
Nichols et al., 2012	Cohort	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	100,00	Low
Balasubramanian et al., 2012	Case-control	Y	U	N	Y	Y	U	U	Y	Y	Y	-----	60,00	Moderate
Weller et al., 2014	Cohort	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100,00	Low
Grobe et al., 2013	Cohort	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100,00	Low
Tinhofer et al., 2014	Cohort	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100,00	Low
Kawada et al., 2016	Cohort	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100,00	Low
Chikamatsu et al., 2019	Cross-sectional	U	Y	U	U	U	U	U	Y	-----	-----	-----	25,00	High
Tada et al., 2020	Cohort	Y	Y	U	Y	Y	Y	Y	Y	U	U	Y	72,70	Low

Article 2

Frequency of cancer stem cells plastic subpopulations in oral squamous cell carcinoma fresh tumor specimens

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ABSTRACT

Recent evidence shows that mechanisms of treatment failure, tumor recurrence, and metastasis of oral squamous cell carcinoma (OSCC) are due principally to the patterns of cellular heterogeneity explained by the model of cancer stem cells (CSC). It is also known that there is a link between the CSC phenotype and the epithelial-mesenchymal transition (EMT) process, which explains the greater capacity of migration and metastatic potential of CSCs compared to the other tumor cells. In this context, the purpose of this prospective study was to quantify the CSC subpopulations in OSCC in large amounts of fresh tumor tissue samples from patients, evaluating the association of the biological properties related to stem-like and EMT phenotypes with the invasive and metastatic behavior of OSCC. The expression levels of CD44 and ESA by epithelial tumor cells was analyzed by flow cytometry in fresh tissue samples, and the results were correlated with clinicopathological parameters. There was a correlation between the frequency of the CSC-enriched population ($CD44^+$) with locoregional lymph nodes metastasis, indicating CSCs influence on the OSCC progression. Considering CSC subpopulations, the association between perineural invasion as well as lymph nodes commitment with the frequency of the epithelial ($CD44^+ESA^+$), but not with the mesenchymal ($CD44^+ESA^-$) subpopulation, suggests that a large volume of CSCs with a mesenchymal profile may not be the only requirement for cell invasion and metastasis, but rather a smaller plastic CSC subpopulation that still needs to be further explored.

Keywords: Oral squamous cell carcinoma, epithelial-mesenchymal transition, cancer stem cells, phenotypical plasticity, flow cytometry.

BACKGROUND

Oral squamous cell carcinoma (OSCC) is one of the most common types of cancer worldwide and still results in significantly high incidence and mortality [1]. The prognostic factor with the most significant impact on this disease is the presence of metastasis in cervical lymph nodes [2]; therefore, the ability to predict tumor invasiveness can improve clinical outcomes establishment and influence treatment decisions.

Current studies focus on the biological and molecular properties of tumor-initiating cell subpopulations to improve diagnostic accuracy, develop individualized therapies, and

monitor recurrences. The cancer stem cells (CSCs), distinctly from other tumor cells that compound the heterogeneity of solid tumors, can proliferate extensively, form new tumors, and resist to conventional therapies [3-5]. Additionally, when CSC of the primary tumor mass undergoes the epithelial-mesenchymal transition (EMT), they invade the surrounding tissue, gain access to lymphatic or blood vessels, and then survive transport to exit from vessels and invade a new tissue site [5, 6]. During this critical process in the invasion-metastasis chain, CSCs exhibit cellular and molecular changes, alternating between epithelial and mesenchymal phenotypes and expressing different markers throughout the stages [7, 8].

The phenotypic plasticity of CSCs has been studied in OSCC. In 2011, Biddle et al. identified two distinct CSC subpopulations in OSCC: $CD44^{+}ESA^{high}$ proliferative non-EMT CSCs (epithelial CSC), and $CD44^{high}ESA^{low/-}$ migratory and metastatic CSCs (post-EMT CSC). By EMT and the reverse process of mesenchymal-epithelial transition (MET), cells were able to switch between these two phenotypes to reconstitute the cellular heterogeneity characteristic of CSCs [9]. In a later study, they were able to show that increased ability to undergo EMT/MET, characterizing the phenotypic plasticity, underlies increased CSC therapeutic resistance within both subpopulations [10]. In a recently published study, Amor et al. characterized *in vitro* and *in vivo* cell behavior of $CD44^{high}ESA^{high}$ and $CD44^{high}ESA^{low}$ sub-fractions from an OSCC cell line. The $CD44^{high}ESA^{high}$ epithelial phenotype showed higher proliferation and holoclone forming capacity than the $CD44^{high}ESA^{low}$ mesenchymal subpopulation, which migrated and invaded more [11].

Thus, since CSCs in EMT are often studied in cell lines and animal models but not representatively *in vivo*, this study expands the analysis to fresh tumor tissues, aiming to quantify and characterize CSCs subpopulations in OSCC samples assessing the frequency of CSC and EMT phenotypes with the clinicopathological characteristics.

MATERIAL AND METHOD

This study was approved (CAAE: 44985615.1.0000.5417) by the local ethics committee for research using human participants at University of São Paulo Medical

School. The patient recruitment began in January 2018 and was completed in January 2020.

Sample collection

Forty-eight OSCC patients from the Institute of Cancer of São Paulo (ICESP; University of São Paulo) were submitted to a punch biopsy right after diagnosis and before treatment started. Inclusion criteria were: patients over 18 years of age, HIV-negative, and without a history of hepatitis. The fresh specimens, measuring about $3mm^2$, were stored at 4°C in supplemented basal culture medium (DMEM/F12, 10% fetal bovine serum) with 4% antibiotics until the processing, no longer than 24 hours.

Fresh tissue digestion

Immediately after receipt, the tumor samples were washed with PBS, minced with a sterile scalpel, and incubated in an enzymatic solution composed of Collagenase type III (75 U/ml; MP Biomedicals) and Dispase (2 mg/ml; Gibco) at 37°C for 2 hours, under gentle agitation. Then, trypsin EDTA (Gibco) was added, remaining at 37°C under gentle agitation for another 15 minutes, or until complete cell clusters dissociation. After inactivation of the enzyme activity by culture medium, the cell suspension was centrifuged, and the pellet was resuspended in PBS to label for flow cytometry.

Flow cytometry

Initially, the cell suspension was filtered through a nylon mesh (Cell Strainer, 70 µm, BD Biosciences) to remove clusters. The Human BD Fc Block™ (BD Biosciences) was used to block potential non-specific antibodies staining caused by Fc receptors. The cells were then incubated with the conjugated antibodies (BD Biosciences) at 4°C for 40 minutes in the dark. The exclusion of dead cells was made using Fixable Viability Stain (FVS; BD Horizon™) or DAPI. Isotype controls were performed for fluorescence compensation, and unstained cells were used to define the equipment voltages and design of the gating strategy. The flow cytometry analysis was performed using BD FACSAria™ Fusion Cell Sorter and BD FACSDiva™ 8.0 software (BD Pharmigen™).

Two distinct multicolor panels were used throughout the project, both with the same targets (**Figure 1-A)**. The flow cytometry analysis started with the selection based on cell size and granularity (**Figure 1-B)**, followed by the single cells selection to exclude doublets (**Figure 1-C)**, and the live cells selection from negative/low expression of the cell viability marker (**Figure 1-D).** The viable cells then underwent by the epithelial tumor cells selection based on β4-Integrin (CD104) positive expression and absence of CD45 expression (**Figure 1-E)**, to, finally, be subdivided by the expression of CD44 and ESA (**Figure 1-F)**.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6 software (GraphPad Software Inc.) and jamovi software version 1.6 (The jamovi project). The data did not show the normality of the variances (Shapiro-Wilk test), so they were compared using Spearman test (for ordinal variables) and Mann-Whitney U or Kruskal-Wallis test (for nominal variables). A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

The study included 48 patients with OSCC at the time set of pathologic diagnosis, before surgical resection or starting any treatment. The age range included patients from 26 to 87 years, predominantly male (75%), and most consumers of alcohol (77.08%) and tobacco (81.25%). The frequency of patients who declared their skin color as "white" was 56.25%, followed by "brown" with 35.42%, and "black" by 8.33%.

The sub-sites of oral tumors included, in descending order, the tongue (43.75%), gingiva (16.67%), retromolar trigone (14.58%), the floor of mouth (10.42%), buccal mucosa (8.33%), and hard palate (6.25 %). The differentiation grade was moderate in the largest portion of patients (64.58%), followed by well-differentiated (22.92%) and poorly differentiated (4.17%) tumors. The respective information of 4 patients (8.33%) was not specified in the medical records. This information and others such as perineural invasion, angiolymphatic invasion, and TNM staging are described in **Figure 3**.

Among the epithelial cells selected by the $CD104^{+}CD45^{-}$ phenotype, the percentage of $CD44^{+}$ epithelial cells ranged widely from 0.23% to 82.63% (median 18.5%**;** interquartile

range 8.03 – 43.2%). Sub-fractioning this $CD44^+$ subpopulation by epithelial-specific antigen (ESA; also known as CD326 or EpCAM) expression, the range of $CD44^+ESA^+$ cells was from 0% to 52.08% (median 7.7%**;** interquartile range 1.57 – 30%) of the epithelial cells, while $CD44^+ESA^-$ cells corresponded from 0 to 56.64% (median 5.3%**;** interquartile range 1.51 – 13.4%). The frequency of cell subpopulations in each tumor tissue sample analyzed is detailed in the **Figure 2**. Using Spearman's correlation test, both the $CD44^+$ population and its subpopulation with epithelial phenotype ($CD44^+ESA^+$) exhibited a directly proportional correlation with pN stage, that classifies the involvement of locoregional lymph nodes (p=0.02). The subpopulation with the mesenchymal phenotype ($CD44^+ESA^-$) was positively correlated with the degree of differentiation of the tumors (p=0.03). The Mann-Whitney test showed a significant difference between the frequency of $CD44^+ESA^-$ cells and alcohol consumption (p=0.03), while the percentage of $CD44^+ESA^+$ cells was related to perineural invasion (p=0.02). Although not statistically significant, there was a trend toward a high frequency of $CD44^+ESA^-$ cells with gender (p=0.051), age (p=0.069), and tobacco consumption (p=0.077). The **Figure 3** shows all results performed by the statistical tests.

DISCUSSION

The OSCC significantly improved 5-year overall survival up to 70% in the last decades, presumably because of advances in imaging and therapy. Perhaps also because of the high diagnostic accuracy, there was an increase in the rate of distant metastases detection [12], which further reinforces the importance of studying the phenotypic plasticity of cells involved in tumor development, growth, and spread. Considering the inter and intratumoral heterogeneity, in addition to genetic and epigenetic factors of each OSCC patient, purifying CSCs from the whole tumor to perform molecular analyses tends to be a more efficient way of investigating this subpopulation and better predict tumor behavior.

The first studies on phenotypic plasticity of CSCs were certainly performed *in vitro* due to the range of problems to fresh tumor samples analysis. Obstacles include the scarcity of tissue available, difficulties in the isolation of live cells, and variation, within and between tumors, of genetic and phenotypic cellular properties [13]. Although it is possible to differentiate tumor cells by histological analysis of paraffin tissue, flow cytometry allows

the analysis of large anatomical pieces that are enzymatically dissociated, generating data from millions of cells in a single analysis [14]. Furthermore, it is possible to separate live cells, avoid unspecific markings, and analyze different markers through multicolor panels. In this study, a significant amount of 48 fresh tumor tissue samples were prospectively analyzed in order to build a real representative scenario of the frequency of subpopulations of CSCs in OSCC.

Joshua et al. performed flow cytometry in head and neck squamous cell carcinoma (HNSCC) samples after single cells dissociation to analyze $CD44^+$ cells. Negative selection by using a cocktail of stromal/inflammatory cell markers called "lineage markers" (anti-CD2, CD3, CD10, CD16, CD18, CD31, CD64, and CD140b) was used to exclude nontumor cells. However, no epithelial cell marker was adopted, or any dead cell exclusion was applied to guarantee the absence of unspecific marking [14]. Recently, Patil used magnetic separation to isolate the $CD44^+$ population after enzymatic digestion of HNSCC tumors, followed by flow cytometry to confirm purity. No other markers were used to positively or negatively select the tumor cells [15]. In the present study, cytometry was designed to minimize the risk of non-specific staining, selecting only live cells after Fc receptor inhibitors were used. Then, tumor cells were isolated from fibroblasts and other stromal cells using positive selection with CD104 (epithelial marker, also known as β4-integrin) since the single positivity to CD44 could lead to wrong estimates of the size of the $CD44^+$ tumor cell subpopulation [14]. Negative selection was also used to exclude lymphocytes by the absence of CD45 expression (lymphocyte common antigen).

In 2006, Prince et al. showed that even a small population of $CD44^+$ tumor cells from HNSCC fresh tumor samples gave rise to new tumors after xenotransplant, reproducing the original tumor heterogeneity [16]. Although it cannot be considered that all $CD44^+$ cells are CSCs, it is possible to state that the subpopulation exhibiting the $CD104^+CD45^-CD44^+$ profile is enriched with CSCs, validating the correlation analysis between the frequency of cells with potential stem profile and data clinicopathological. In the study of Joshua et al. described above, the range of frequency of Lin^-CD44^+ cells was 0.4% to 81% in twenty-two HNSCC samples evaluated [14]. Lin^-CD44^+ cell frequency was significantly correlated with T classification and subsequent failure. Patients with a Lin-$CD44^+$ cell frequency

<15% had a higher overall survival rate than patients with a $Lin^{-}CD44^{+}$ cell frequency >15% [14]. In OSCC, Emich et al. reported an unpublished work with a range of $CD44^{+}$ cells between 3% and 97.7% [17]. Accordingly, our methodology, including forty-eight OSCC samples, was able to detect very similar values, ranging from 0.23% to 82.63% of $CD104^{+}CD45^{-}CD44^{+}$ cells. The correlation of frequency of this subpopulation with locoregional lymph nodes metastasis was directly proportional, indicating CSCs influence on the OSCC progression.

Among the CSCs, plastic subpopulations can be isolated regarding the level of expression of ESA since this important epithelial marker has its expression altered according to the stage of the cell under EMT process [9, 10, 18]. In OSCC immortalized cell lines, subclones with a mesenchymal morphology showed high expression of CD44 and low expression of ESA, while the more epithelial phenotype is characterized by high expression of ESA [9, 11]. Analyzing seven OSCC specimens, Biddle et al. sorted $CD44^{+}$ subpopulation after discrimination of β4-Integrin positivity. The epithelial profile $CD44^{+}ESA^{high}$ presented frequencies between 3.6% and 52.3%, while the migratory/metastatic post-EMT subpopulation $CD44^{high}ESA^{low/-}$ ranged from 0.37% and 38.5% [10]. In the present study, the $CD44^{+}ESA^{-}$ (mesenchymal) subpopulation presented a frequency rate ranging from 0 to 56.64%, and was correlated with the degree of tumor differentiation and alcohol consumption. The $CD44^{+}ESA^{+}$ (epithelial) subpopulation frequency ranged from 0% to 52.08% and the statistical analysis showed the highest frequencies correlated with the highest rate of perineural invasion and locoregional lymph nodes commitment. Interestingly, both strong invasiveness and metastasis indicators were previously associated with CSCs mesenchymal phenotype subpopulation [9]. Indeed, when Amor et al. inoculated $CD44^{high}$ cells in mice, $CD44^{high}ESA^{low}$ (mesenchymal) subpopulation generated fewer and smaller tumors compared with $CD44^{high}ESA^{high}$ (epithelial) cells [11]. Therefore, our data suggest that a large volume of CSCs with a mesenchymal profile may not be the only requirement for cell invasion and metastasis, but rather a subpopulation with potential for resistance and phenotypic plasticity.

There is still a long way to determine the applicability of markers of plastic CSC subpopulations for predicting patient therapeutic response. Future studies with CSCs from OSCC fresh tumors should include additional markers such as CD24 (heat-stable antigen), CD29 (β1-integrin), ALDH1 (aldehyde dehydrogenase), and CD133 (prominin-1) to refine the selection of the CSC population [19-23]. The analysis of CD44 variant isoforms expressions should also be considered since some were associated in HNSCC with advanced T stage, regional and distant metastasis, perineural invasion, and radiation failure [24]. Knowing that large multicolor panels for flow cytometry may be unfeasible, we suggest prior fluorescence-activated cell sorting of viable $CD104^{+}CD45^{-}$cells, followed by post-analysis using flow cytometry or RT-qPCR for CSC and EMT markers. Thus, it will be possible to thoroughly explore the frequency of subpopulations representing poor prognosis and treatment failure in OSCC.

CONFLICT OF INTERESTING

The authors declare no conflict of interest.

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A

	Panel 1	Panel 2
Viability marker	FITC (FVS520)	APC (FVS660) or DAPI
Integrin β4 (CD104)	PE	PE
CD44	PerCP-Cy5.5	APC
ESA (CD326)	BV605	BB515
CD45	APC-H7	APC-H7

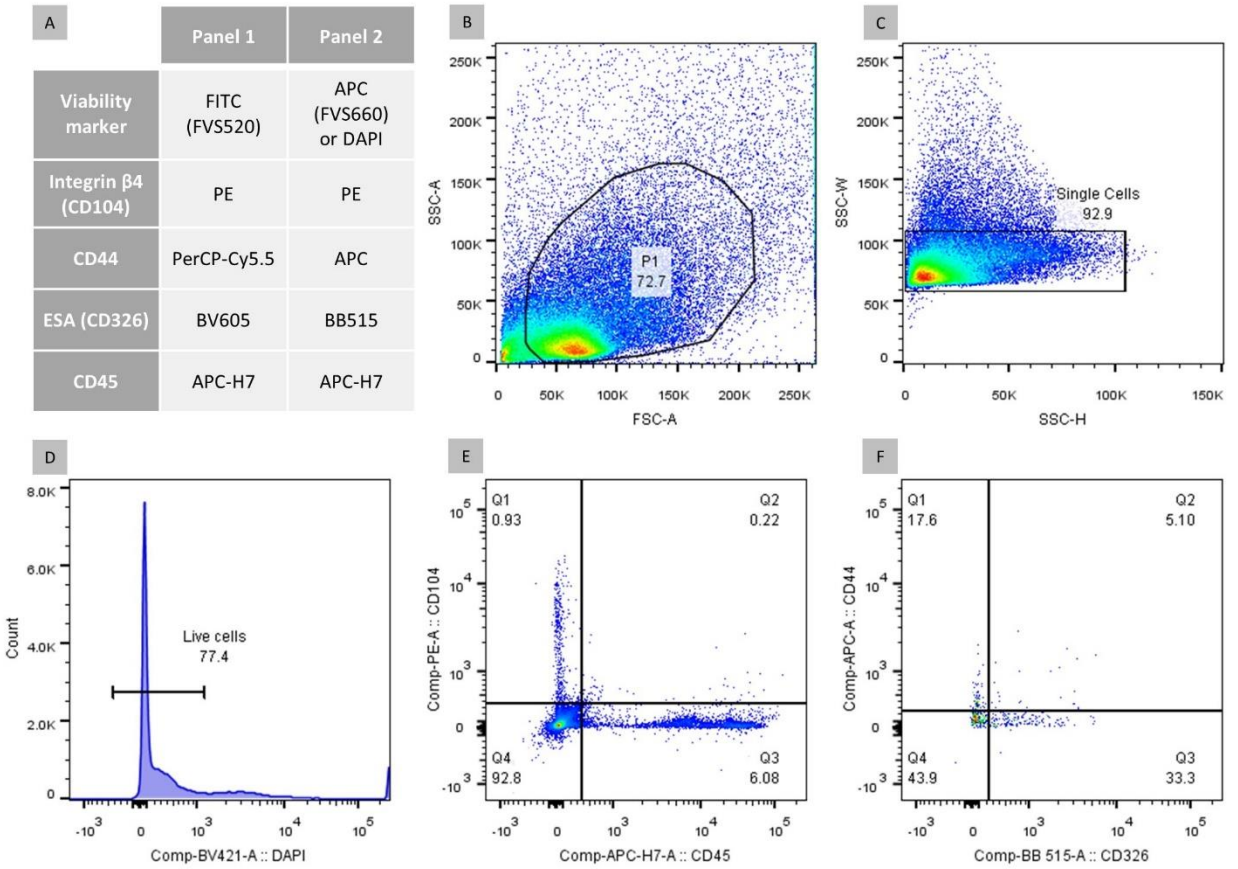

Figure 2. Multicolor flow cytometry panels and the flow chart of the gating strategy used for identification of different subpopulations of CSCs by flow cytometry. (A) Multicolor flow cytometry panels used in flow cytometric analysis. (B-F) Analysis example using the fluorescence panel 2, where (B) is the cell size and granularity first selection; (C) is the doublets exclusion; (D) is the live cells selection; (E) is the selection of epithelial tumor cells ($CD104^{+}CD45^{-}$); and (F) shows the expression of CD44 (y-axis) and ESA (x-axis) where population Q1 represents $CD104^{+}CD45^{-}CD44^{+}ESA^{-}$ cells, and population in Q2 represents $CD104^{+}CD45^{-}CD44^{+}ESA^{+}$ cells.

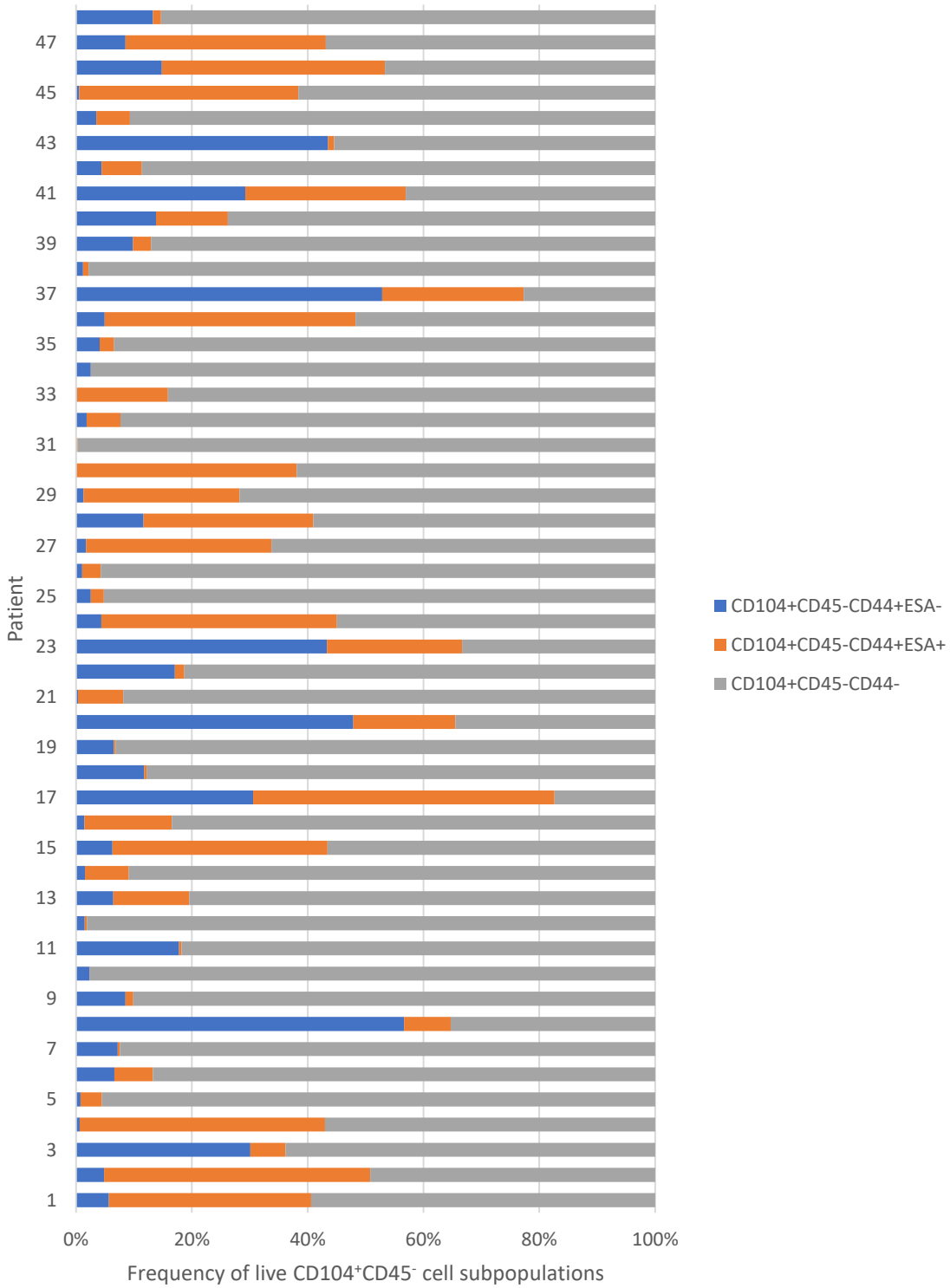

Figure 3. Frequency of cell subpopulations in each tumor tissue sample analyzed by flow cytometry. All subpopulations were first gated on viable cells, then only $CD104^{+}CD45^{-}$ cells are selected for CD44 and ESA expression analyses. The percentage of $CD104^{+}CD45^{-}CD44^{+}$ cells can be considered by the sum of the $CD104^{+}CD45^{-}CD44^{+}ESA^{+}$ and $CD104^{+}CD45^{-}CD44^{+}ESA^{-}$ subpopulations.

Figure 4. Correlation of cell subpopulations frequency to clinicopathological parameters.

Clinicopathological parameters	Category	n	%	P value		
				$CD104^+CD45^-CD44^+$	$CD104^+CD45^-CD44^+ESA^-$	$CD104^+CD45^-CD44^+ESA^+$
Gender	Female	12	25.00	0.581†	0.051†	0.897†
	Male	36	75.00			
Age	<60	25	52.08	0.698†	0.069†	0.378†
	≥60	23	47.92			
Ethnicity/skin color	Black	4	8.33	0.984‡	0.983‡	0.945‡
	Brown	17	35.42			
	White	27	56.25			
Alcohol	No	11	22.92	0.296†	*0.031†	0.981†
	Yes	37	77.08			
Tobacco	No	9	18.75	0.938†	0.077†	0.499†
	Yes	39	81.25			
Sub-site	Hard palate	3	6.25	0.649‡	0.400‡	0.896‡
	Buccal mucosa	4	8.33			
	Floor of mouth	5	10.42			
	Retromolar trigone	7	14.58			
	Gingiva	8	16.67			
	Tongue	21	43.75			
Lymphatic invasion	Yes	13	27.08	0.524†	0.456†	0.986†
	No	20	41.67			
	NS	15	31.25			
Angio invasion	Yes	11	22.92	0.667†	0.938†	0.457†
	No	21	43.75			
	NS	16	33.33			
Perineural invasion	Yes	17	35.42	0.189†	0.682†	*0.020†
	No	15	31.25			
	NS	16	33.33			
T classification	1	2	4.17	0.823§	0.586§	0.461§
	2	4	8.33			
	3	11	22.92			
	4	31	64.58			
N classification	0	18	37.50	*0.023§	0.486§	*0.021§
	1	4	8.33			
	2	13	27.08			
	3	13	27.08			
M classification	0	44	91.67	0.528†	0.928†	0.602†
	1	4	8.33			
Differentiation grade	Poor	2	4.17	0.098§	*0.036§	0.898§
	Moderate	31	64.58			
	Well	11	22.92			

NS, Not specified; † calculated by Mann-Whitney U test; ‡ calculated by Kruskal-Wallis test; § calculated from the Spearman rank correlation.
*Indicates a statistically significant difference.

3 Discussion

Advances in imaging and therapy probably improved the OSCC significantly 5-year overall survival up to 70% in the last decades. Perhaps also because of the high diagnostic accuracy, there was an increase in the rate of distant metastases detection (AMIT; YEN; LIAO; CHATURVEDI *et al.*, 2013), which further reinforces the importance of studying the phenotypic plasticity of cells involved in tumor development, growth, and spread. Considering the inter and intratumoral heterogeneity, in addition to genetic and epigenetic factors of each OSCC patient, purifying CSCs from both the whole tumor and peripheral blood tends to be a more efficient way of investigating this subpopulation and its reflect in the disease outcome.

The first studies on phenotypic plasticity of CSCs were certainly performed *in vitro* due to the range of problems to fresh tumor samples analysis. Obstacles include the scarcity of tissue available, difficulties in the isolation of live cells, and variation, within and between tumors, of genetic and phenotypic cellular properties (COSTEA; GAMMON; KITAJIMA; HARPER *et al.*, 2008). Similarly, CTC detection remains technically challenging, especially in OSCC patients, due to low concentrations in whole blood. For example, in one background of millions of blood cells, a single tumor cell can be detected (ALIX-PANABIÈRES; PANTEL, 2013). Therefore, the most accurate detection methodologies for rare cells are highly dependent on the chosen technology and biological markers.

Joshua et al. performed flow cytometry in twenty-two HNSCC samples to analyze $CD44^+$ cells with a cocktail of stromal/inflammatory cell markers called "lineage markers" (anti-CD2, CD3, CD10, CD16, CD18, CD31, CD64, and CD140b) to exclude non-tumor cells. The range of frequency of Lin^-CD44^+ cells was 0.4% to 81%, and significantly correlated with T classification, subsequent failure, and overall survival (JOSHUA; KAPLAN; DOWECK; PAI *et al.*, 2012). In OSCC, Emich et al. reported an unpublished work with a range of $CD44^+$ cells between 3% and 97.7% (EMICH; CHAPIREAU; HUTCHISON; MACKENZIE, 2015). Our methodology, including forty-eight OSCC samples, was able to detect very similar values, ranging from 0.23% to 82.63% of $CD104^+CD45^-CD44^+$ cells. The correlation of frequency of this subpopulation with locoregional lymph nodes metastasis was directly proportional, indicating CSCs influence on the OSCC progression.

ESA (CD326; also known as EpCAM) is an important epithelial marker and has its expression altered according to the stage of the CSCs within the EMT process (BIDDLE; GAMMON; LIANG; COSTEA *et al.*, 2016; BIDDLE; LIANG; GAMMON; FAZIL *et al.*, 2011; BROWN; SANKPAL; GILLANDERS, 2021). In OSCC immortalized cell lines, subclones with a mesenchymal morphology showed high expression of CD44 and low expression of ESA, while the more epithelial phenotype is characterized by high expression of ESA (AMÔR; BUZO; ORTIZ; LOPES *et al.*, 2021; BIDDLE; LIANG; GAMMON; FAZIL *et al.*, 2011). Biddle et al. sorted $CD44^+$ subpopulation from seven OSCC specimens after the selection of β4-Integrin positivity. The epithelial profile $CD44^+ESA^{high}$ presented frequencies between 3.6% and 52.3%, while the migratory/metastatic post-EMT subpopulation $CD44^{high}ESA^{low/-}$ ranged from 0.37% and 38.5% (BIDDLE; GAMMON; LIANG; COSTEA *et al.*, 2016). In the present study, the $CD44^+ESA^-$ (mesenchymal) CSC subpopulation presented a frequency rate ranging from 0 to 56.64%, correlated with the degree of tumor differentiation and alcohol consumption. The $CD44^+ESA^+$(epithelial) CSC subpopulation frequency ranged from 0% to 52.08%, and the statistical analysis showed the highest frequencies correlated with the highest rate of perineural invasion and locoregional lymph nodes commitment. Although the literature reports that CSCs mesenchymal phenotype subpopulation was associated with invasiveness and metastasis (BIDDLE; LIANG; GAMMON; FAZIL *et al.*, 2011), and our data show no association with these cells and lymph nodes metastasis, is plausible to suggest that maybe a small subset of the metastatic subpopulation ($CD44^+ESA^-$) with the potential for resistance could be enough for OSCC recurrence and metastasis.

The first approach to isolate CTCs among all blood cells of patients with cancers of epithelial origin was targeting epithelial antigens (ESLAMI-S; CORTÉS-HERNÁNDEZ; ALIX-PANABIÈRES, 2020). The negative selection based on depletion of $CD45^+$ cells combined with the positive selection of epithelial markers (mainly ESA) has been used widely for a long time in HNSCC. However, the potentially more metastatic CTCs remain undetected by ESA-based enrichment methods due to their low/absence expression during the EMT state. In many cancers, this mesenchymal-like subpopulation of CTCs was correlated with poor outcomes, indicating that this phenotypic change is advantageous in circulation and other sites (ALIX-PANABIÈRES; MADER; PANTEL,

2017; DASGUPTA; LIM; GHAJAR, 2017). Tada et al. evaluated four epithelial markers (ESA, EGFR, c-Met, and KRT19) for CTC detection in forty-four HNSCC blood samples, and only three patients were positive for all four markers, 1 for three markers, 7 for two markers, and 17 for one marker (TADA; TAKAHASHI; KUWABARA-YOKOBORI; SHINO *et al.*, 2020). Therefore, it is still not possible to state that a single epithelial marker can be used to isolate all possible phenotypes of CTCs, including mesenchymal CTCs undergoing EMT.

The use of enrichment and detection methodologies that do not depend on the expression of surface epithelial markers has been encouraged to avoid failure to detect CTCs undergoing EMT, including the mesenchymal-like subpopulations (GORGES; TINHOFER; DROSCH; RÖSE *et al.*, 2012; RUSHTON; NTELIOPOULOS; SHAW; COOMBES, 2021). Weller et al. used density enrichment and detected CTCs with epithelial properties ($CK^+/Ncad^-/CD45^-$ and $CK^+/CD133^-/CD45^-$) as well as CTCs with mesenchymal features ($N\text{-}cad^+/CK^-/CD45^-$) and CTCs with both phenotypic characteristics ($N\text{-}cad^+/CK^+/CD45^-$) by immunofluorescence (WELLER; NEL; HASSENKAMP; GAULER *et al.*, 2014). Balasubramanian et al. used centrifugation and immunomagnetic negative selection enrichment based on CD45 marker expression. They evaluated two distinct profiles of CTCs with mesenchymal markers expression by immunofluorescence; one positive for an epithelial marker ($CK^+/Vimentin^+$/EGFR, CD44, or $N\text{-}cad^+$) and another one negative ($CK^-/Vimentin^+$/EGFR, CD44, or $N\text{-}cad^+$), suggesting that the CTCs have undergone the EMT process (BALASUBRAMANIAN; LANG; JATANA; MILLER *et al.*, 2012). Furthermore, positivity for CD44 reinforces that these EMT cells may be circulating CSCs.

There are still many gaps to fill when it comes to detecting subpopulations of CSCs in EMT. The phenotypic plasticity of CSCs found in primary tumors and peripheral blood of OSCC patients shows how variable the panel of markers used in future methodologies must be. In order to refine the selection of the CSC population before other analyses, the next studies with OSCC fresh tumors should include additional markers such as CD24, CD29, CD133 (GENG; GUO; WANG; LI *et al.*, 2013; GHUWALEWALA; GHATAK; DAS; DEY *et al.*, 2016; LU; LU; LI; ZHU *et al.*, 2014; OKAMOTO; CHIKAMATSU; SAKAKURA;

HATSUSHIKA *et al.*, 2009), and CD44 variant isoforms expressions (WANG; WONG; DE HEER; XIA *et al.*, 2009). With more accurate data and an assertive molecular profile of CSCs in EMT, it can be extrapolated for the analysis of CTCs, since the great immediate potential of CTC detection is certainly disease monitoring (PANTEL; SPEICHER, 2016). Future personalized medicine strategies for OSCC patients will be based on liquid biopsy, with subsequent and rapidly reported molecular analysis of CTCs, ensuring detection of the most aggressive profiles of CSCs in the bloodstream to predict metastases and determine therapeutic interventions.

4 Conclusion

An accurate capture and identification methodology must be established to detect CTCs in OSCC patients, considering the different possible cellular phenotypes in the disease progression. Analyzing CSCs subpopulations in EMT from the primary tumor is a major step towards establishing this profile. Considering the wide range of CSCs with no expression of ESA reported in our study, the detection of CTCs based on this marker becomes highly inaccurate. Efficient molecular markers have to be elected to improve the detection of CTCs in OSCC progression, including targets to select circulating CTCs undergoing EMT and investigate their CSC phenotype.

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Appendix

DECLARAÇÃO DE USO EXCLUSIVO DE ARTIGO EM DISSERTAÇÃO/TESE

Declaramos estarmos cientes de que o trabalho "*Detection methodologies of circulating tumor cells in oral squamous cell carcinoma: a systematic review*" será apresentado na Tese da aluna Nathália Martins Lopes, e que não foi e nem será utilizado em outra dissertação/tese dos Programas de Pós-Graduação da FOB-USP.

Bauru, 24 de novembro de 2021.

Nathália Martins Lopes	Assinatura
Rafael Carneiro Ortiz	Assinatura
Rebeca Barros Nascimento	Assinatura
Nádia Ghinelli Amôr	Assinatura
Maria Fernanda Setúbal Destro Rodrigues	Assinatura
Flávia Caló Aquino Xavier	Assinatura
Camila de Oliveira Rodini	Assinatura

Annexes

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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Isolamento e caracterização de células-tronco de carcinoma epidermóide de boca em tecido fresco e sangue periférico: papel da plasticidade fenotípica no processo de metástase

Pesquisador: Camila de Oliveira Rodini Pegoraro

Área Temática:

Versão: 2

CAAE: 76480517.0.0000.0065

Instituição Proponente: FUNDACAO FACULDADE DE MEDICINA

Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 2.348.103

Apresentação do Projeto:

O projeto foi bem fundamentado com bases em dados atualizados da literatura. O texto é apresentado de forma clara e objetiva. Não envolve participação estrangeira e nem há participação de outros serviços/ divisões do HCFMUSP. Trata-se de um estudo prospectivo com finalidade acadêmica "DOUTORADO" da aluna Nathalia Martins Lopes com orientação da Profa. Dra. Camila de Oliveira Rodini Pegoraro. As amostras serão coletadas no Serviço de Cirurgia de Cabeça e Pescoço do Instituto do Câncer do Estado de São Paulo (ICESP) e processadas no Departamento de Ciências Biológicas da Faculdade de Odontologia de Bauru – Universidade de São Paulo (FOB-USP). O projeto tem apoio financeiro da FAPESP na forma de Auxílio à Pesquisa Jovem Pesquisador (Processo nº 2013/07245-9). O pesquisador apresenta carta de anuência da Faculdade de Odontologia de Bauru da Universidade de São Paulo e parecer consubstanciado favorável do ICESP (Registro NP 1156/17 de 12 de julho de 2017). O cronograma de execução apresentado é adequado.

Objetivo da Pesquisa:

"Objetivo primário"

Quantificar e caracterizar células-tronco de câncer (CSC) e macrófagos em carcinoma epidermóide de boca (CEB), tanto em tecido tumoral fresco, quanto em amostras de sangue periférico de pacientes com estágio avançado da doença, buscando-se avaliar a associação das propriedades

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biológicas relacionadas ao fenótipo tronco tumoral e de transição epitélio-mesenquimal, com o comportamento invasivo e metastático do CEB.

“Objetivos secundários”

Considerando-se, então, que a subpopulação de CSC que sofreu o processo de transição epitéliomesenquimal (TEM) é a responsável pela geração de metástase linfonodal, bem como compõe uma fração das células tumorais circulantes, podendo ser identificada pelos níveis de expressão de proteínas relacionadas com pluripotência e fenótipo mesenquimal, têm-se os seguintes objetivos específicos:

1. Caracterizar as CSC presentes no CEB, por meio da quantificação da proporção dessas células em amostras tumorais individuais, utilizando-se o nível de expressão dos marcadores CD44 e ESA, por citometria de fluxo;
2. Correlacionar a proporção da subpopulação de CSC com os dados clinico-patológicos dos tumores (pTNM, grau de diferenciação, estadiamento) e sobrevida dos pacientes;
3. Obter linhagens celulares primárias derivadas dos tumores, a serem posteriormente investigadas quanto ao seu potencial como modelo experimental apropriado para análise de CSC;
4. Identificar CSC e com fenótipo de TEM dentre as CTCs em casos de CEB avançado, por meio de técnicas de imunofluorescência e qRT-PCR;
5. Caracterizar o perfil dos macrófagos isolados de lesões de CEB e analisar sua influência no fenótipo e capacidade invasiva de CSC em ensaios in vitro.

Avaliação dos Riscos e Benefícios:

“Riscos”:

São previstos os mesmos riscos inerentes ao procedimento de biópsia para diagnóstico ao qual o paciente passará de acordo com o protocolo clínico do hospital. Há previsão de desconforto e risco mínimo durante a coleta de sangue, no entanto, enfatizamos que são os mesmos existentes para as outras coletas de sangue que já tenham sido realizadas.

“Benefícios”:

Tratando-se de uma pesquisa laboratorial, a mesma não traz benefícios diretos e imediatos para o indivíduo voluntário. Porém, cabe ressaltar que os resultados da pesquisa poderão contribuir para o esclarecimento sobre os mecanismos que regulam a metástase do câncer de boca e ajudar no desenvolvimento de novos tratamentos para o carcinoma epidermóide de boca.

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Comentários e Considerações sobre a Pesquisa:

Trata-se de um estudo prospectivo com finalidade acadêmica "DOUTORADO" da aluna Nathalia Martins Lopes com orientação da Profa. Dra. Camila de Oliveira Rodini Pegoraro. As amostras serão coletadas no Serviço de Cirurgia de Cabeça e Pescoço do ICESP e processadas no Departamento de Ciências Biológicas da FOB-USP. O projeto tem apoio financeiro da FAPESP na forma de Auxílio à Pesquisa Jovem Pesquisador.

Considerações sobre os Termos de apresentação obrigatória:

Os documentos obrigatórios foram apresentados de forma satisfatória.

Recomendações:

Sem recomendações.

Conclusões ou Pendências e Lista de Inadequações:

APROVADO

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_977744.pdf	20/10/2017 14:58:36		Aceito
Outros	Carta_resposta_CEP_FMUSP_2.pdf	20/10/2017 14:57:49	Nathália Martins Lopes	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_final_CORRIGIDO.pdf	20/10/2017 14:53:18	Nathália Martins Lopes	Aceito
Declaração de Instituição e Infraestrutura	Carta_de_anuencia_FOBUSP.pdf	06/09/2017 15:00:29	Nathália Martins Lopes	Aceito
Outros	Formulario_CEP_FMUSP_anexo.pdf	06/09/2017 13:37:25	Nathália Martins Lopes	Aceito
Folha de Rosto	folhaDeRosto_assinada.pdf	06/09/2017 13:34:44	Nathália Martins Lopes	Aceito
Outros	Termo_de_outorga_aditivo_2013_072459.pdf	23/08/2017 14:47:59	Camila de Oliveira Rodini Pegoraro	Aceito
Outros	Parecer_NP_ICESP.pdf	23/08/2017 08:57:12	Camila de Oliveira Rodini Pegoraro	Aceito
Outros	Anuencia_Biobanco.pdf	22/08/2017 15:40:01	Nathália Martins Lopes	Aceito
TCLE / Termos de Assentimento /	TCLE.pdf	22/08/2017 15:36:14	Nathália Martins Lopes	Aceito

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Continuação do Parecer: 2.348.103

Justificativa de Ausência	TCLE.pdf	22/08/2017 15:36:14	Nathália Martins Lopes	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO PAULO, 25 de Outubro de 2017

Assinado por:
Antonio de Padua Mansur
(Coordenador)

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Nathália Martins Lopes <nalopes@usp.br>

Submission Confirmation

O Oncology Editorial Office <em@editorialmanager.com> 15 de novembro de 2021 23:07
Responder a: O Oncology Editorial Office <support@elsevier.com>
Para: Nathalia Martins Lopes <nalopes@usp.br>

Re: "Detection methodologies of circulating tumor cells in oral squamous cell carcinoma: a systematic review"
Nathalia Martins Lopes; Rafael Carneiro Ortiz; Rebeca Barros Nascimento; Nádia Ghinelli Amôr; Maria Fernanda Setúbal Destro Rodrigues; Flávia Caló Aquino Xavier; Camila Oliveira Rodini
Review Article

Dear Ms. Martins Lopes,

Your submission entitled "Detection methodologies of circulating tumor cells in oral squamous cell carcinoma: a systematic review" has been received by journal Oral Oncology

You will be able to check on the progress of your paper by logging on to Editorial Managers as an author. The URL is https://www.editorialmanager.com/oo/.

Your username is: nalopes@usp.br
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Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

Editorial Manager
Oral Oncology

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