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Research Paper

Epithelial-mesenchymal transition related to bone invasion in oral squamous cell carcinoma

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ABSTRACT

Introduction: Bone invasion is an important prognostic factor in oral squamous cell carcinoma, leading to a lower survival rate and the use of aggressive treatment approaches. Epithelial-mesenchymal transition (EMT) is possibly involved in this process, because it is often related to mechanisms of cell motility and invasiveness. This study examined whether a panel of epithelial-mesenchymal markers are present in cases of oral squamous cell carcinoma with bone invasion and whether these proteins have any relationship with patients' clinical-pathological parameters and prognostic factors.
Methods: Immunohistochemical analysis of E-cadherin, *twist*, vimentin, TGFβ1, and periostin was performed in paraffin-embedded samples of 62 oral squamous cell carcinoma cases.
Results: The analysis revealed that most cases (66%) presented with a dominant tumor infiltrative pattern in bone tissue, associated with lower survival rates, when compared with cases with a dominant erosive invasion pattern (P = 0.048). Twenty-seven cases (43%) expressed markers that were compatible with total or partial EMT at the tumor-bone interface. There was no association between evidence of total or partial EMT and other demographic or prognostic features. E-cadherin-positive cases were associated with tobacco smoking (P = 0.022); vimentin-positive cases correlated with tumors under 4 cm (P = 0.043). *Twist*expression was observed in tumors with a dominant infiltrative pattern (P = 0.041) and was associated with the absence of periostin (P = 0.031).
Conclusion: We observed evidence of total or partial EMT in oral squamous cell carcinoma bone invasion. The transcription factor *twist* appears to be involved in bone invasion and disease progression.

1. Introduction

Head and neck cancer is a significant global public health challenge, of which oral squamous cell carcinoma (OSCC) is the most prevalent subtype [1]. Patients with advanced disease usually present with severe involvement of the principal structures of the oral cavity, including the submucosa, salivary glands, and bone; regional metastasis is also a common feature. However, surgical treatment of these cases has functional, aesthetic, psychological, and social consequences [2,3]. Despite new therapeutic strategies, many patients have a limited response to treatment, with metastasis and frequent recurrence leading to poor survival rates—the 5-year survival rate is approximately 50% [4].

Bone invasion is a significant poor prognostic factor in OSCC [2]. Gross tumor invasion of the maxilla or mandibular bone can be detected by imaging, but in many cases, microinvasions and small invasions are detected only in the final histological examination [5,6]. Further, a systematic review from 2013 found that the detection of superficial bone invasion using imaging methods depends on the interpretation by and experience of the physician and is

Abbreviations: OSCC, oral squamous cell carcinoma; EMT, epithelial-mesenchymal transition.

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not always accurate [6]. Artificial intelligence technologies are promising tools for improving the screening, imaging, and early detection of oral cancer [7]. Also, the measurement of bone invasion markers is needed to improve its diagnosis and understand the mechanisms of tumor infiltration.

The molecular mechanisms of bone invasion in OSCC are poorly understood. The release of cytokines and growth factors is elevated in the extracellular matrix, accompanied by osteoclast recruitment and bone resorption. Once neoplastic cells invade the bone, a favorable environment for growth and cell division is created, initiating a cycle that accelerates and maintains bone destruction [8]. This microenvironment might also alter neoplastic cell behavior, inducing epithelial-mesenchymal transition (EMT), which can perpetuate this cycle.

EMT is a complex and reversible biological process that comprises phenotypic and transient cellular changes, in which a polarized epithelial cell undergoes biochemical changes to assume a mesenchymal cell phenotype; this phenomenon enhances motility and invasiveness, the resistance to apoptosis, and the production of extracellular matrix components and enzymes [9,10]. In turn, these characteristics promote aggressive behavior and drug resistance in tumor cells, perhaps explaining the invasion and destruction of bone tissue. Changes in *snail*, *ZEB,* and *twist* protein expression, which are regulated by transcription factors, could be used as biomarkers to detect and evaluate EMT. The loss of epithelial proteins (E-cadherin, cytokeratins, and laminins) and a gain in mesenchymal proteins (N-cadherin, vimentin, and fibronectin) are hallmarks of EMT [11]. Further, these proteins are prognostic markers, based on their association with survival and metastasis [12,13].

The function of EMT in soft tissue infiltration has been studied extensively in oral SCC, but its involvement in bone invasion remains unknown. Thus, the objective of this retrospective study was to determine whether there is any evidence of EMT in bone invasion in OSCC. Further, we examine whether there is any relationship between the expression of EMT proteins and clinicopathological and prognostic parameters in the cases that studied. To this end, we analyzed 5 proteins that are involved in EMT: E-cadherin (an epithelial protein), vimentin (a mesenchymal protein), TGFβ1 (a growth factor that activates EMT), twist (a transcription factor that is essential for activating and modulating EMT), and periostin (involved in bone metabolism and EMT) [14,15].

2. Methods

This retrospective study comprised surgical specimens from the Surgical Pathology Department of Hospital das Clínicas, University of São Paulo Medical School. The study was approved by our institutional review board (protocol number 2.201.788). Data on all patients with OSCC who underwent surgical resection between 2008 and 2015 were obtained from the hospital's records (n = 493). Then, we selected patients with unequivocal bone invasion, as reported in their histopathological records (n = 130). The slides of their surgical resections were analyzed, and cases with insufficient material for immunohistochemical analysis were excluded (68 cases excluded). The final sample size for the proposed analysis included 62 cases.

Hematoxylin-and-eosin-stained slides were re-evaluated by 2 pathologists to confirm bone invasion and classify the histological patterns as erosive or infiltrative. The erosive pattern comprised an extensive and well-delimited invasive front in the bone tissue, with osteoclastic resorption and fibrosis. The infiltrative pattern presented as nests and projections of tumoral cells and penetration of the Harversian system. Cases with features of both patterns were classified as mixed [2]. For such mixed cases, the most predominant pattern was deemed to be the final classification, as illustrated in Fig. 1.

3. Immunohistochemistry

Paraffin-embedded tissue blocks were sectioned into 3-μm-thick slices and placed on glass slides. The slides were deparaffinized and rehydrated through xylene and descending grades of alcohol. Antigen retrieval was performed according to Table 1. The specimens were incubated in 10 volumes of hydrogen peroxide for 10 min to block endogenous peroxidase.

The sections were then incubated with the primary antibodies for 16 h at 4 °C (dilutions listed in Table 1). Antigen-antibody complexes were visualized using the Novolink™ detection system (Novocastra, Leica Biosystems Newcastle Ltd, Ref. RE7159/RE7161) and incubated with 3,3′ diaminobenzidine tetrachloride (DAB Substrate Kit – Cell Marque, 6600 Sierra College Blvd. CA95677 USA). The sections were then counterstained with Carazzi's hematoxylin, dehydrated in ascending grades of alcohol and xylene, and mounted with a glass coverslip and xylene-based mounting media. The positive controls for each antibody are described in Table 1; the reactions were considered to be valid only if the control tissue slides were properly stained.

Semiquantitative analysis of the immunoexpression patterns of proteins in the tumor-bone interface was performed to classify the cases as partial or total EMT. The immunoexpression of each protein in the tumor-bone interface was evaluated by 2 pathologists and scored positive—when over 10% of tumor cells showed positivity of any intensity (weak, medium, or high)—or negative. Cases that were E-cadherin-negative, *twist*-positive, and positive for 2 or 3 other proteins were classified as being consistent with total EMT. Cases that were positive for E-cadherin, *twist,* and 2 or 3 other proteins were considered to be compatible with partial EMT.

4. Statistical analysis

The sociodemographic and histological characteristics, immunohistochemical results, and clinical data were compared by chi-squared test or Fisher's exact test, as appropriate. Overall survival was evaluated by Kaplan-Meier method, and curves were compared by log-rank test. SPSS® version 21.0 (SPSS® Inc; Illinois, USA) was used in the statistical analysis, and the probability of an α or type I error was set to 5% or less ($P \leq 0.05$).

5. Results

The clinical, demographic, and histopathological characteristics of the 62 cases in the study sample are summarized in Table 2. A predominant infiltrative bone invasion pattern was seen in 41 cases (66%), and 21 cases (34%) presented with a dominant erosive bone invasion pattern. Fig. 1 A and B illustrate the aspects of these invasion patterns. Patients with a dominant infiltrative pattern experienced significantly worse survival (log-rank 0.048), with a median survival rate of 39.6 months for dominant erosive versus 26.1 months for dominant infiltrative (P = 0.048, log-rank test), as shown in Fig. 2.

Of the 62 OSCC cases, 43% (27) presented with criteria that were compatible with EMT—total EMT was detected in 17 cases, and partial EMT was seen in 10 cases at the tumor-bone interface. Fig. 3 illustrates the immunohistochemical patterns of a case that was judged to be total EMT.

Briefly, E-cadherin was considered positive when it surrounded the tumor cell membrane or was expressed in the cytoplasm. It was detected in tumor islands in infiltrative and invasive invasion patterns in 39 cases. Vimentin was not detected in OSCC tumor

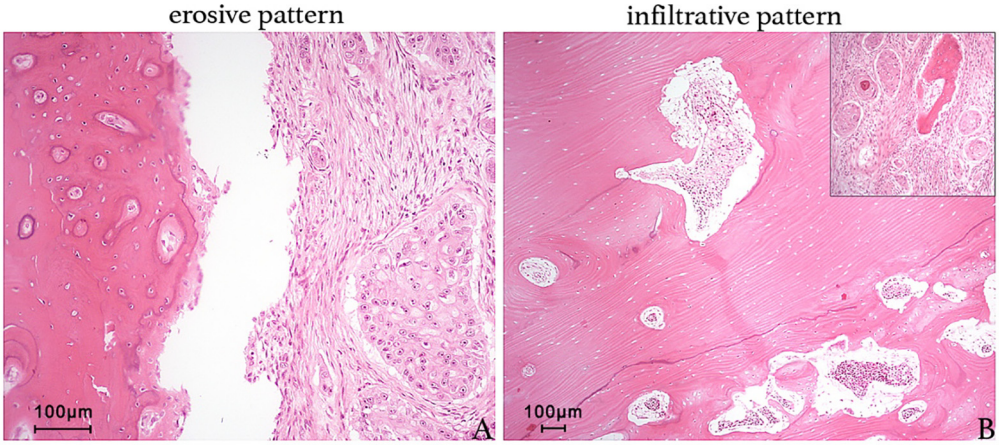

Fig. 1. Histopathological aspects of OSCC bone invasion patterns. A: OSCC with erosive bone invasion pattern: observe tumor islands close to bone trabecula, which is surrounded by plump osteoclasts. H&E, original optical magnification ×100. B: OSCC with infiltrative bone invasion pattern: note OSCC tumor islands intermingling bone trabeculae with varied aspects of resorption. H&E, original optical magnification X40.

Table 1
Antibodies.

Antibody	Manufacturer	Dilution	Positive Control	Clone	Antigen retrieval
VIMENTIN	Abcam (ab28028)	1:300	Kidney/Lymph node	Mouse monoclonal (VIM3B4)	Tris-EDTA, pH 9.0. Steamer 45′
PERIOSTIN	Novus Biologicals (NBP1-30042)	1:250	Brain	Rabbit polyclonal	Trypsin 20′
TGFB1	Santa Cruz Biotechnology Inc. (sc-146)	1:4000	Stomach	Rabbit polyclonal	Tris-EDTA, pH 9.0. Steamer 45′
TWIST	LS Bio (LS C191858)	1:1500	Colon tumor	Mouse monoclonal (10E4E6)	Citrate, pH 6.0. Steamer 45′
E-CADHERIN	Cell Marque (CMC24631050)	1:300	Skin	Rabbit monoclonal (EP700Y)	Trypsin 20′

Table 2
Epidemiological and clinicopathological characteristics of the 62 patients surgically treated for OSCC with bone invasion.

Variable		N (%)
Gender	Male	47 (75%)
	Female	15 (25%)
Tobacco Smoking		40 (65%)
Alcohol Consumption		35 (56%)
Positive Resection Margins		16 (26%)
Perineural Invasion		48 (77%)
Angiolymphatic/vascular Invasion		20 (32%)
Lymph node metastasis		34 (55%)
Extracapsular Spread		18 (59,2%)
Degree of Differentiation	Good	15 (24%)
	Moderate	40 (65%)
	Poor	7 (11%)
Locoregional Recurrence		33 (53%)
Distant Metastasis		8 (13%)
Lymph Node Metastasis		34 (55%)
Death		36 (58%)
Radiotherapy		30 (48%)
Chemotherapy		17 (27%)
Median follow-up		21.11 months

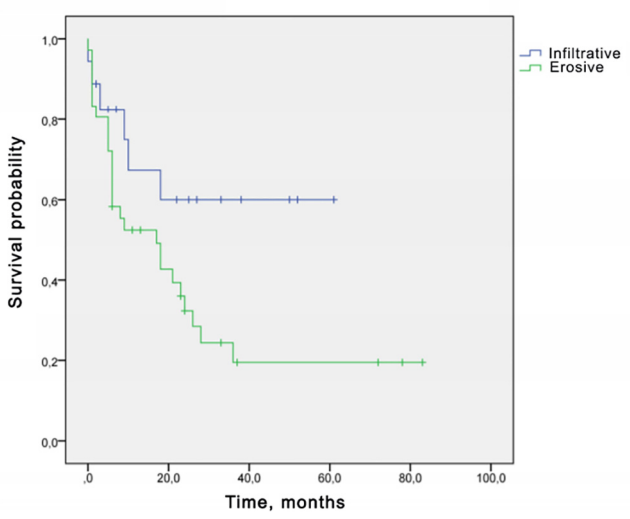

Fig. 2. Overall survival curve for OSCC patients considering dominant bone invasion pattern. Lower survival rate in dominant infiltrative pattern (log-rank 0.048, erosive mean – green = 39.64; infiltrative mean – blue = 26.113). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

islands in any type of bone invasion. It was positive in stromal cells and in osteoclasts that surrounded the resorbed bone trabeculae and was detected focally in 28 cases of OSCC. The OSCC tumor islands in the erosive or infiltrative pattern showed widespread Twist positivity. This factor was expressed in 51 cases and nuclear in neoplastic cells. Periostin was positive in several tumor islands in tumors with erosive or infiltrative bone invasion patterns in 39 cases. Finally, TGF-beta was detected in 32 specimens; this factor was expressed in tumor islands in invasion patterns or erosive or infiltrative bone invasion patterns, including in neoplastic cells near the bone trabeculae.

Table 3 lists the statistical results between clinical and histopathological parameters and EMT status. There was no association between evidence of total or partial EMT and demographic, clinical, or histopathological parameters. Individual analysis of each protein revealed that E-cadherin-positive cases were more frequent in tobacco smokers (P = 0.022). There was a link between vimentin expression in tumors that were smaller than 4 cm (P = 0.043). OSCC cases with a dominant infiltrative pattern correlated with *twist* expression (P = 0.041). Positive *twist* expression was also associated with the absence of periostin (P = 0.031).

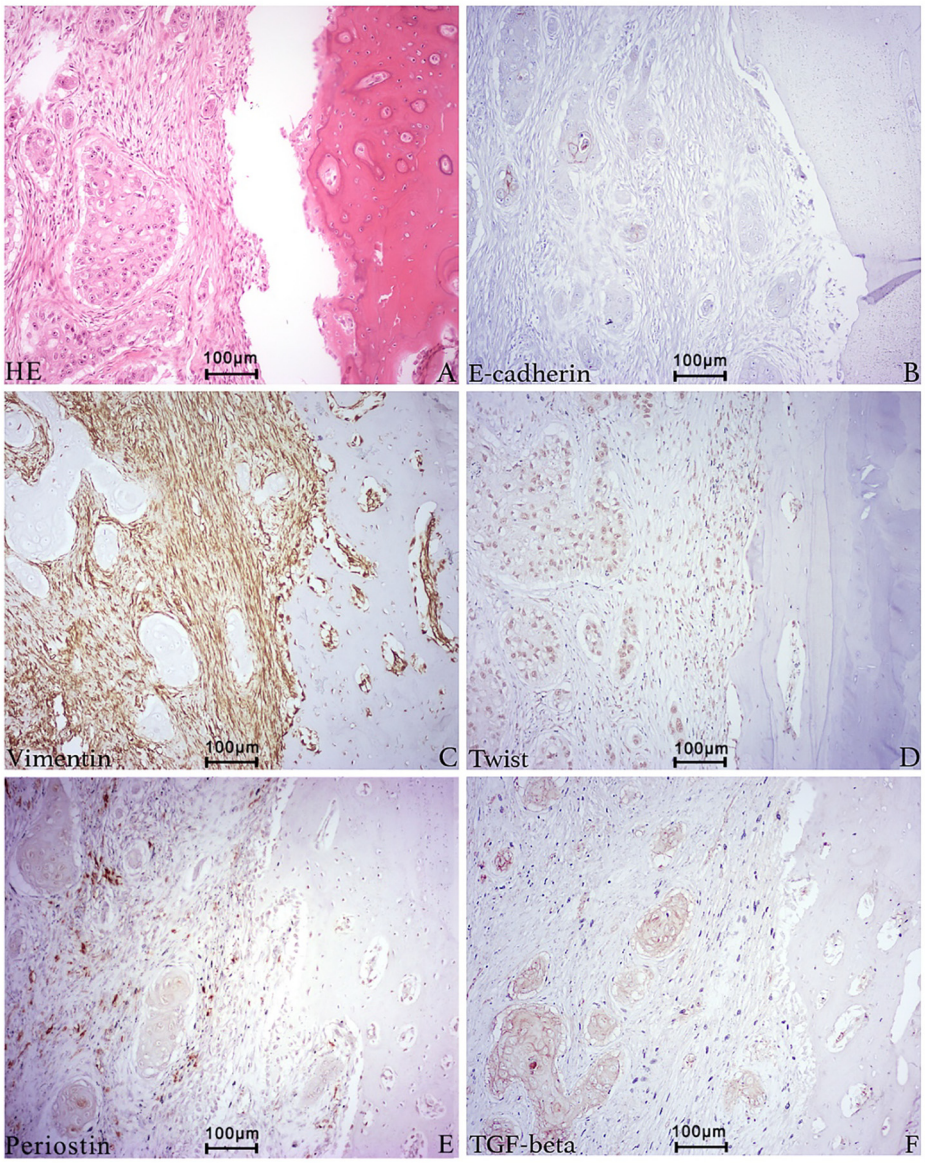

Fig. 3. Examples of EMT proteins in serial sections of oral squamous cell carcinoma with erosive bone invasion. A: Histological aspect of erosive pattern of oral squamous cell carcinoma. Note the close relationship between tumor islands and bone trabecula. Original optical magnification ×100. B: Absence of E-cadherin in erosive bone invasion pattern of OSCC. Note the expression of the protein only around the cell membranes at the center of some tumor islands. Original optical magnification ×100. C Vimentin expression in osteoclasts and stromal cells, but negative in OSCC islands. Original optical magnification ×100. D: Twist widespread nuclei positivity in OSCC tumor islands. Original optical magnification ×100. E: Scarce tumor islands positive for periostin. Original optical magnification ×100. F: The expression of TGF-beta 1 is present in tumor islands. Original optical magnification ×100.

6. Discussion

OSCC is the most common head and neck cancer. Despite the advances in medicine and research, there are many challenges in the treatment and survival of OSCC patients, which can be overcome by determining its molecular mechanisms. Bone invasion is an important factor that impacts the staging and prognosis of the disease, even in small tumors [16]. When bone invasion is present, aggressive treatment approaches are usually necessary, including extensive surgeries and adjuvant therapies [3]. EMT is a process that promotes aggressive and invasive tumor cell behavior. Thus, the involvement of the molecular mechanisms of EMT in bone invasion must be considered in this complex scenario.

We and other groups have analyzed the histological patterns of bone invasion, divided into 3 types: erosive, infiltrative, and mixed. We found an association between the dominant infiltrative pattern and a lower overall survival rate, suggesting that the infiltrative pattern is a prognostic factor, indicating a poor clinical outcome [17]. Cases with an erosive pattern had a better prognosis compared with the infiltrative pattern, with longer survival rates, as shown in our results. This finding could be explained by the surgical complexity that is encountered in obtaining clear margins in cases with an infiltrative pattern, due to the greater irregularity of the tumor invasion front [2,18]. The histological pattern of bone invasion and its prognostic value remains debated in the literature—eg, cortical versus medullary invasion—as is the interpretation of which the invasion is classified, considering heterogeneous criteria [19].

EMT is a dynamic process that can be detected focally or transiently at various stages of tumor progression in response to specific changes in the tumor microenvironment. The concept of a partial EMT has added new layers of complexity, one of which con-

Table 3
Comparisons of clinicopathological and prognostic factors with tumors with and without EMT.

Variable		EMT +	EMT −	p-value
Gender	Male	9 (22.5%)	31 (77.5%)	0.716 (F)
	Female	4 (26.7%)	11 (73.3%)	
Tobacco Smoking	Negative	1 (11.2%)	8 (88.8%)	1.000 (F)
	Positive	5 (12.5%)	35 (87.5%)	
Alcohol Consumption	Negative	2 (14.3%)	12 (85.7%)	1.000 (F)
	Positive	4 (11.5%)	31 (88.5%)	
Resection Margins	Free	37 (80.4%)	9 (19.6%)	0.725 (F)
	Positive	4 (25%)	12 (75%)	
Perineural Invasion	Negative	3 (23.1%)	10 (76.9%)	1.000 (F)
	Positive	10 (20.5%)	39 (79.5%)	
Angiolymphatic Invasion	Negative	9 (21.5%)	33 (78.5%)	1.000 (F)
	Positive	4 (20%)	16 (80%)	
Node Metastasis	pN0	8 (34.8%)	15 (65.2%)	0.100 (X)
	pN1	1 (11.2%)	8 (88.8%)	
	pN2a	0	0	
	pN2b	2 (18.2%)	9 (81.8%)	
	pN2c	0	13 (100%)	
	pN3	0	2 (100%)	
Distant Metastasis	Present	2 (25%)	6 (75%)	0.595 (F)
	Absent	6 (14%)	37 (86%)	
Adjuvant Chemotherapy	Positive	2 (11.8%)	15 (88.2%)	1.000 (F)
	Negative	4 (12.5%)	28 (87.5%)	
Adjuvant Radiotherapy	Positive	5 (16.7%)	25 (83.3%)	1.000 (F)
	Negative	3 (13.1%)	20 (86.9%)	

F: Exact Fisher's test; X: chi-square test.

cerns the appropriate tool and procedures for evaluating EMT and its function in cancer progression and metastasis [20,21]. Our analysis used a protein panel to characterize EMT in bone invasion. We also focused on the tumor-bone interface, given the differences in the biomolecular characteristics of the tumor invasive front and tumor mass.

We found that 27% of cases (n = 17/62) fulfilled the criteria for classification as being compatible with total EMT and that 16% of cases (n = 10/62) were compatible with partial EMT at the tumor-bone interface. Despite this important morphological information, immunohistochemical analysis only allows static evaluation of the disease, which is considered a limitation in assessing EMT in tissue specimens. Nevertheless, our work provides the basis for *in vitro* functional studies. Also, EMT could be not identified in certain slides in our sample, possibly developing in another stage of the disease or area of the tumor.

A study that used an animal model of bone invasion discussed the possibility of tumor cells undergoing EMT, triggered by bone tissue [22], and we found evidence of this process in our study. EMT can downregulate or lead to nonfunctional expression of adhesion proteins, improving cell survival mechanisms and aggressive tumor behavior [23].

Loss of E-cadherin, an important epithelial cell adhesion protein, is a hallmark of EMT, associated with increased expression of mesenchymal cytoskeletal proteins, such as N-cadherin and vimentin. These changes result in the acquisition of a mesenchymal phenotype, migratory capacity, and invasive properties. E-cadherin and vimentin expression has been used to identify a partial or total EMT and has been reported as potential prognostic markers in OSCC [24,25]. In our study, we did not find any association between these 2 proteins and clinical parameters.

Most cases in our cohort lacked E-cadherin, suggesting that contact between tumor cells and bone tissue downregulates E-cadherin. Certain cases presented with characteristics of a partial EMT, with positive E-cadherin expression. Because this protein promotes cell adhesion, in specific situations, its presence can confer advantages to neoplastic cells, such as in collective cell migration, increasing the likelihood of neoplastic cell survival [26]. Tumor cells with a partial EMT coexpress epithelial and mesenchymal proteins. Several *in vivo* studies have demonstrated the existence of partial EMT tumor cells and their higher metastatic potential, based on their ability to disseminate in collective migration and as single cells [21].

Another study reported that E-cadherin can be expressed in an aberrant form, forming clusters of adherens junctions, which are linked to actin filaments, increasing cell motility [26]. E-cadherin can also stimulate osteoclast differentiation; thus, its expression by tumor cells or in the tumor microenvironment can enhance bone resorption [22].

We found a notable association between E-cadherin-positive cases and tobacco smoking; there are no studies that have analyzed E-cadherin expression between OSCC smokers and nonsmokers patients. A previous study examined this protein in patients with high alcohol consumption, tobacco smoking, and no oral lesions compared with OSCC patients [27], reporting high E-cadherin expression in patients with no lesions and loss of this protein in OSCC patients. New studies are necessary to determine whether the microenvironment in bone invasion promotes molecular changes that affect E-cadherin expression.

The expression of periostin and TGFβ1 in most of our cases also implicates EMT in bone invasion. These 2 proteins are closely involved in the induction and regulation of this mechanism [11]. Periostin is considered a suitable target for immunotherapy [14]. One report associated periostin overexpression in oral SCC with metastasis [28], but this link was not confirmed in our study. TGFβ1 also participates in bone resorption by activating osteoclasts [29]. TGFβ-related proteins have been examined as predictive markers and therapeutic targets of bone invasion in oral SCC [30].

Twist is a well-established transcription factor that is associated with EMT. *Twist* expression is an essential criterion for identifying EMT in tumor tissue samples and has been linked to metastasis in head and neck SCC [15]. We found that positive *Twist* expression was associated with an infiltrative invasion pattern and with the absence of periostin. These findings suggest that *Twist* participates in the interaction and progression of OSCC in bone tissue and, specifically, the microenvironment—a relationship that has not been examined in the literature.

7. Conclusions

Our analysis has revealed the presence of EMT markers in bone invasion of oral squamous cell carcinoma, implicating their involvement in bone invasion, a hypothesis that should be tested in future studies. No significant associations were found between cases with evidence of EMT and clinicopathological or prognostic parameters. Our findings on *Twist* expression suggest that this transcription factor has an important relationship with progression of the disease to bone tissue.

CRediT authorship contribution statement

Jaqueline Vaz Vanini: Conceptualization, Investigation, Writing – original draft. **Leonardo Kenji Sakaue Koyama:** Investigation, Formal analysis. **Leandro Luongo de Matos:** Writing – review & editing, Formal analysis. **José Martins Figueredo Junior:** Writing – review & editing. **Claudio Roberto Cernea:** Formal analysis. **Cibele Pidorodeski Nagano:** Data curation. **Cláudia Malheiros Coutinho-Camillo:** Supervision. **Ricardo Hsieh:** Supervision, Methodology, Resources. **Silvia Vanessa Lourenço:** Conceptualization, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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