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Cortical microarchitecture and remodeling-associated gene expression related to oral cancer prognosis

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Abstract: The objective of this study was to assess the remodeling-associated gene expression in the mandible of patients diagnosed with oral squamous cell carcinoma (OSCC), investigating the cortical microarchitecture, and their influence on disease-free survival (DFS) and overall survival (OS) rates. A total of twenty-four patients who underwent mandibulectomy for OSCC treatment had two bone fragments harvested from the mandible for gene expression (RANK, RANKL, OPG, and SOST), and microarchitecture analysis, including bone volume, surface, mineral density, degree of anisotropy, and fractal dimension. The prognosis of the patients was assessed. The results revealed that RANK, RANKL, and SOST were predominantly downregulated, while OPG was completely downregulated. Tumors located adjacent to the posterior region of the mandible ($p = 0.02$), with a bone mineral density below 1.03 g/cm^3 HA ($p = 0.001$), and a bone volume less than 86.47% ($p = 0.03$) were associated with poor outcomes. In conclusion, bone-remodeling-associated genes exhibited downregulation in the cortex of the mandible in OSCC patients. Additionally, the tumor's location within the mandible, bone volume, and cortical bone mineral density were identified as factors impacting DFS.

Keywords: Neoplasms; Squamous Cell; Bone and Bones; Survival analyis.

Introduction

Oral squamous cell carcinoma (OSCC) belongs to a group of solid tumors that originates from the epithelium of the oral cavity. This disease has the potential for metastatic evolution, leading to a poor prognosis.[1,2] Bone invasion by OSCC has major implications for tumor staging[3] and influences treatment decisions, particularly when considering surgical options for bone resection. OSCC evolves and/or invades the mandibular or maxillary bone through an erosive, infiltrative, or mixed pattern way, which is indicative of tumor behavior.[4,5] The infiltrative pattern is characterized by the formation of irregular nests and projections of neoplastic cells into the bone, including tumor penetration into the Haversian system and/or cancellous bone. Conversely, the erosive form of bone invasion is defined by a sharp transition between the tumor and the bone, with noticeable osteoclastic bone resorption on the surface, accompanied by fibrosis along the tumor/bone interface.

The mixed form combines both patterns of invasion in the same bone/tumor interface.[6,7] Besides the bone invasion site and the regions in its proximity, our understanding of the bone in the surrounding areas, which has not been directly affected by the invasion process, remains limited.

Bone remodeling-associated genes are crucial in the remodeling process and play a key role in bone invasion by malignant tumors, as well as other neoplastic or reactive lesions.[8,9] The receptor activator of nuclear factor κB (RANK) and its ligand (RANKL) are particularly important for the formation of bone-resorbing osteoclasts. Osteoclast-mediated bone resorption is a key step in the process of bone invasion by oral SCCs.[6,10] Certain cytokines (*e.g.*, TNFα and PTHrP) induce the expression of receptor activator of NF-κB ligand (RANKL) or suppress osteoprotegerin (OPG) in oral squamous cell carcinoma (OSCC) cells and cancer stromal cells, promoting osteoclastogenesis. OSCCs create a suitable microenvironment for osteoclastogenesis to regulate the balance of RANKL and OPG.[11]

Another protein related to remodeling and metabolism is sclerostin, which is produced by osteocytes and encoded by the SOST gene. Sclerostin acts as a paracrine regulator of WNT signaling and in the activity of osteoblasts and osteoclasts on bone surfaces.[12,13] Beyond its role in bone remodeling, sclerostin could be considered an independent prognostic factor for OSCC patients.[14]

Although the mechanisms underlying bone and tumor crosstalk are partially known, there is still much to uncover. The question is whether there is a relationship between cortical bone and tumors beyond the invasion status and whether it has an impact on the progression and prognosis of OSCC patients. A previous study revealed that the peripheral cortical bone of OSCC lesions had a higher bone surface area compared to the surgical margin. In cases of bone invasion, a loss of anisotropy was observed. Additionally, this same study also examined the expression of RANK, RANKL, OPG, and SOST at these sites, revealing a predominant downregulation of RANKL and SOST.[15]

The relationship between mandible invasion, surgical treatment, prognosis, and survival rates in OSCC patients is complex and intricate. Lesions at lower anatomical subsites (relative to an imaginary plane passing through the base of the retromolar trigone), bone invasion, and lymph nodal spread were prognostic factors that significantly affected the survival of patients with T4b oral cancers.[16] A study comparing the outcomes of marginal and segmental mandibulectomies did not find differences in locoregional recurrence and disease-specific survival rates.[17]

Generally, poor prognosis in OSCC is attributed to multifactorial variables such as margin status (bone and soft tissue), pattern of bone invasion, medullary bone involvement, and nodal status, among others. Furthermore, in a more in-depth analysis of the bone, a small cohort revealed that DFS was associated with higher osteoclast density along the tumor front invading the mandible.[18]

Hence, the question arises as to whether the prognosis of OSCC patients is not only influenced by bone invasion but also by the involvement of the surrounding cortical bone, independent of the invasion status since it forms part of the microenvironment. Is the cortical bone remodeling process altered in the proximity of the tumor beyond the bone/tumor interface? Therefore, the objective of the present study was to evaluate the microarchitecture of the mandibular cortical bone and the gene expression of RANK, RANKL, OPG, and SOST in the mandible of patients diagnosed with OSCC. In addition, we assessed the impact of the expression of these genes involved in bone remodeling and the microarchitectural parameters on disease-free survival (DFS) and overall survival (OS) rates.

Methodology

This prospective, cross-sectional, single-center study enrolled a total of 24 patients treated for OSCC in Brazil. The patients were selected by convenience sampling, and their surgical treatment included either marginal or segmental mandibulectomy. Patients with a history of diseases known to affect bone metabolism (*e.g.*, prior malignancies in the head and neck region treated by radiotherapy, previous use of

bisphosphonates or monoclonal antibodies such as Denosumab®, or long-term (> 3 months) glucocorticoid treatment) were excluded from the study.

After surgical resection, two bone fragments were collected from the mandible cortex: one was immediately frozen in liquid nitrogen for gene expression analysis, and the other was fixed in 70% alcohol for microarchitecture analysis. The collection of bone fragments followed the criteria established in a previous study by Rabelo et al.[15] Briefly, bone fragments were biopsied using a trephine on the surface of the cortical bone, 5 mm away from the bone/tumor interface to ensure they were tumor-free. Bone invasion status was confirmed only when verified information was found in the anatomical and/or imaging reports (histopathology and computed tomography exams). The invasion status was used for comparison of the microarchitectural parameters (with and without confirmed invasion) to identify any structural alterations in the region adjacent to the tumor. This comparison was conducted under two conditions: a) a normal cortical bone 5 mm away from the tumor interface without invasion; and b) a normal cortical bone 5 mm away from the tumor interface with confirmed bone invasion, being both evolvement of cortical and trabecular components.

Survival analyses were calculated based on both microarchitecture and gene expression data. Survival rates were calculated in months from the date of the primary surgery to the date of any evidence of local recurrence or local, regional, or distant metastasis for DFS and to the date of death for OS.

The project was approved by the Institutional Research Ethics Committee (CAAE: 85565618.3.3001.5432; Approval number: 3.195.138). Written informed consent was obtained from all patients, and relevant clinical data and tumor specifications were collected from their medical records. All procedures involving human participants were conducted in accordance with the ethical standards set by the institutional and/or national research committee, as well as the principles outlined in the Declaration of Helsinki from 1964 and its subsequent amendments or comparable ethical standards.

Gene expression: quantitative PCR

The frozen bone fragment was ground while immersed in liquid nitrogen and then homogenized using Precellys®24 (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA isolation was performed using the RNeasy Mini Kit purification protocol (Qiagen, Valencia, USA), followed by quantification using a NanoDrop™ ND-1000 (Thermo Scientific, Waltham, USA), and treatment with DNase-I (Ambion, Grand Island, USA). Complementary DNA (cDNA) was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA). All procedures followed the institutional protocol.[19]

The analysis was performed in two separate periods with a 1.5-year interval. The first analysis included 18 patients. Gene expression was assessed using primer probe sets from Applied Biosystems for the following genes: RANK (HS00921372_m1), RANKL (HS00243522_m1), OPG (HS00900358_m1), and SOST (Sclerostin) (HS00228830_m1). Quantitative real-time polymerase chain reaction (PCR) was performed, and the expression levels were normalized to the human GAPD (GAPDH) endogenous control (4326317E). In the second period, the PCR was carried out following the same protocol, except for the use of a different endogenous control, the human B2 M (beta-2-microglobulin - HS99999907_m1) endogenous control (VIC® / TAMRA Probe, Primer Limited). Expression levels were measured using the ΔΔCt method[20], and the calibration was performed using the mRNA pool of normal bone harvested from the cortex of three healthy patients who underwent surgery of the mandible for odontogenic cyst removal (the bone was removed 5 mm away from the surgical margin). Downregulation was considered when the relative value was lower than 0.5. Normal gene expression was considered when the relative value ranged from 0.5 to 2, and values greater than 2 were considered overexpression.

Three-dimensional cortical bone microarchitecture

Microscopic characterization of the embedded bone samples was performed using three-dimensional bone microarchitecture analysis with microtomography

(μCT, Scanco MicroCT40, Brüttisellen, Switzerland), using a nominal isotropic voxel size of 19 μm (X-ray source: 55 kVp, 144 μA). The following parameters were determined: bone volume (BV/TV), bone surface (BS/TV), degree of anisotropy (DA), cortical porosity (Ct. Po), pore number (Po. N), fractal dimension (FD, #), and bone mineral density (BMD, g/cm^3 HA - hydroxyapatite). FD was assessed using 3 regions of interest (ROIs) in a square format positioned at the cortex in the μCT images, specifically 3 sections at the middle of the cortex. The box-counting method was used on the grayscale images21 of the ROIs, and the FracLac[22] plugin for ImageJ/FIJI (version 1.53q, National Institute of Health, USA) was utilized. The mean value of the 3 regions was obtained for statistical analysis.

Statistical analysis

The Kaplan-Meier estimator, log-rank test, and uni- and multivariable Cox proportional hazard regression model were calculated (after the determination of a single cut-off point). The results were considered significant when $p < 0.05$. Statistical analyses were performed using IBM® SPSS® Statistics 24 software. For comparison between cases with and without bone invasion, normality was tested by the Shapiro-Wilk test, and comparisons were performed by the Mann-Whitney test for BV/TV and the unpaired t-test for BS/BV, BMD, and FD.

Results

Forty-eight bone samples were obtained from 24 patients, following the same criteria in the previous study.[15] Among the patients, 17 were male and 7 were female. The average age was 59.71 years (± 13; median of 62 years). The tumor position in relation to the mandible (in a reference line of an imaginary plane passing through the canine region) revealed that 37.5% (n = 9) were located in the anterior region, while 62.5% (n = 15) were in the posterior region. The primary tumor sites included the gingiva, retromolar region, floor of the mouth (7 cases each), and tongue (3 cases). Bone invasion occurred in 33.3% of the cases.

Regarding histopathology, differentiation grading was: 75% (n = 18) were diagnosed as moderately differentiated, 8.3% (n = 2) as poorly differentiated, and 16.7% (n = 4) as well differentiated. The mean tumor size was 4.1 cm (median of 3.5 cm, ranging from 0.6 to 8.5 cm). Local recurrence/metastasis occurred in 41.6% (n = 10) of the patients, and 20.8% (n = 5) dies during the follow-up period. The mean follow-up time was 40.6 months, ranging from 7 to 85 months.

Cortical bone analysis

None of the cases showed gene overexpression. Most of the cases exhibited downregulation. The normal expression levels for RANK and RANKL were 29.2% and 4.2%, respectively. Downregulation was observed in 41.7% of the cases for RANK and 70.8% for RANKL (7 missing cases for RANK and 6 for RANKL). OPG was downregulated in all cases (100%, 7 cases missing). SOST was downregulated in 75% of the cases, while 8.3% showed normal levels (4 missing cases) (Figure 1). No statistically significant associations were found between gene expression levels and DFS or OS.

Regarding microarchitecture, the characteristics of the mandibular cortex for all 24 patients are shown in Table 1. A comparison between cases with bone invasion (n = 8) and those with no pathological and imaging evidence of bone evolvement and/or erosion (n = 16) did not reveal any significant differences (Table 2) (Figure 2).

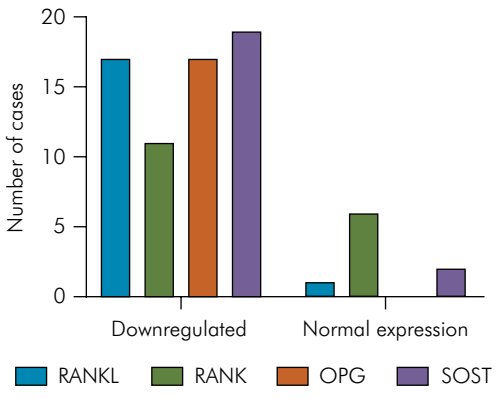

Figure 1. Bar graph showing the number of cases classified as downregulated or with normal expression for the metabolism-associated-genes RANK, RANKL, OPG, and SOST.

Table 1. Cortical microarchitectural parameters evaluated in Microtomography.

Parameters	n	Mean	Median
BV/TV (%)	24	96.71	98.05
BS/BV (mm)	24	4.31	4.19
DA (#)	18	0.77	0.79
Ct.Po (%)	24	3.29	1.94
Pores (n)	18	38.89	32.00
BMD (g/cm³ HA)	24	1.07	1.08
FD (#)	23	1.21	1.23

n (number of samples evaluated for the analyzed parameter). BV/TV bone volume fraction, BS/BV specific bone surface, DA degree of anisotropy, Ct.Po cortical porosity, BMD bone mineral density, FD fractal dimension.

Table 2. Comparison of cortical microarchitecture between cases with and without bone invasion.

Parameters	p	Bone invasion	No bone invasion
BV/TV (%)	0.52	97.18 (93.25; 99.21)	98.05 (96.57; 99.33)
BS/BV (mm)	0.44	3.87 ± 1.18	4.52 ± 2.19
BMD (g/cm³ HA)	0.87	1.07 ± 0.08	1.07 ± 0.05
FD (#)	0.20	1.24 ±0 .07	1.18 ± 0.12

BV/TV (Median, 25%; 75% percentiles). BS/BV, BMD, and FD (Mean ± Standard-deviation).

Disease-free survival (DFS)

The tumor relationships with the mandible, the cortical bone volume, and the bone mineral density were all revealed to be significant predictive variables for DFS. Tumors at oral sites located in relation to the posterior part of the mandible were associated with the worst DFS ($p = 0.02$). Regarding cortical microarchitectural parameters, bone volume lower than 86% ($p = 0.03$) and bone mineral density below 1.03 g/cm³ HA ($p = 0.001$) were also related to recurrence. Multivariate Cox regression analysis revealed that the bone mineral density revealed a hazard ratio of 8.5 for the worst DFS outcome when it was less than 1.03 g/cm³ HA ($p = 0.009$). The tumor's relation to the mandible revealed a tendency for a worse prognosis ($p = 0.057$), with a hazard ratio of 7.43 for the worst DFS for the posterior mandible (Figure 3).

Overall survival (OS)

Patients' OS was estimated, and a survival rate of 79.2% was found. The estimated Kaplan-Meier average survival, in months, was 67.69% (CI =54.82–80.55). No bone parameter and remodeling-related gene expression status were related to OS. Bone invasion

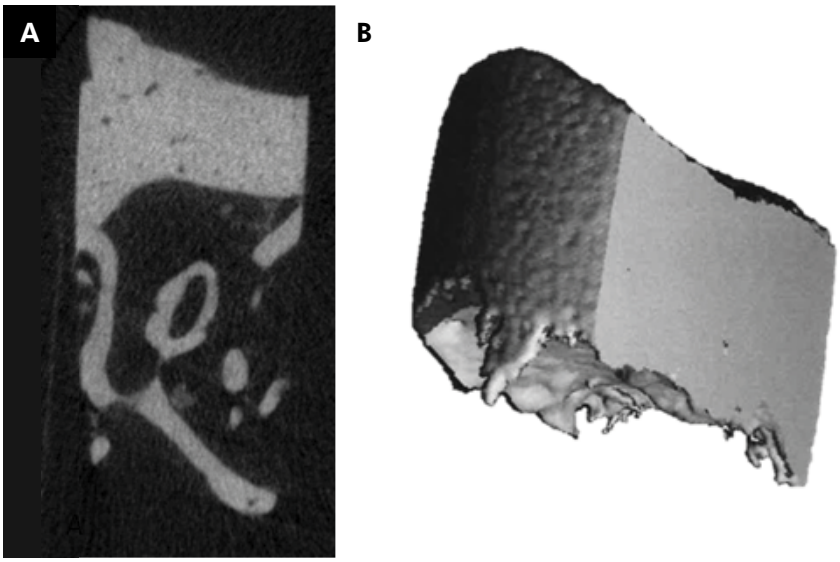

Figure 2. Representative image of the analyzed bone microarchitecture. A) μCT gray-level slice of the sample. B) Reconstructed 3D model of the cortex analyzed for microarchitecture parameters.

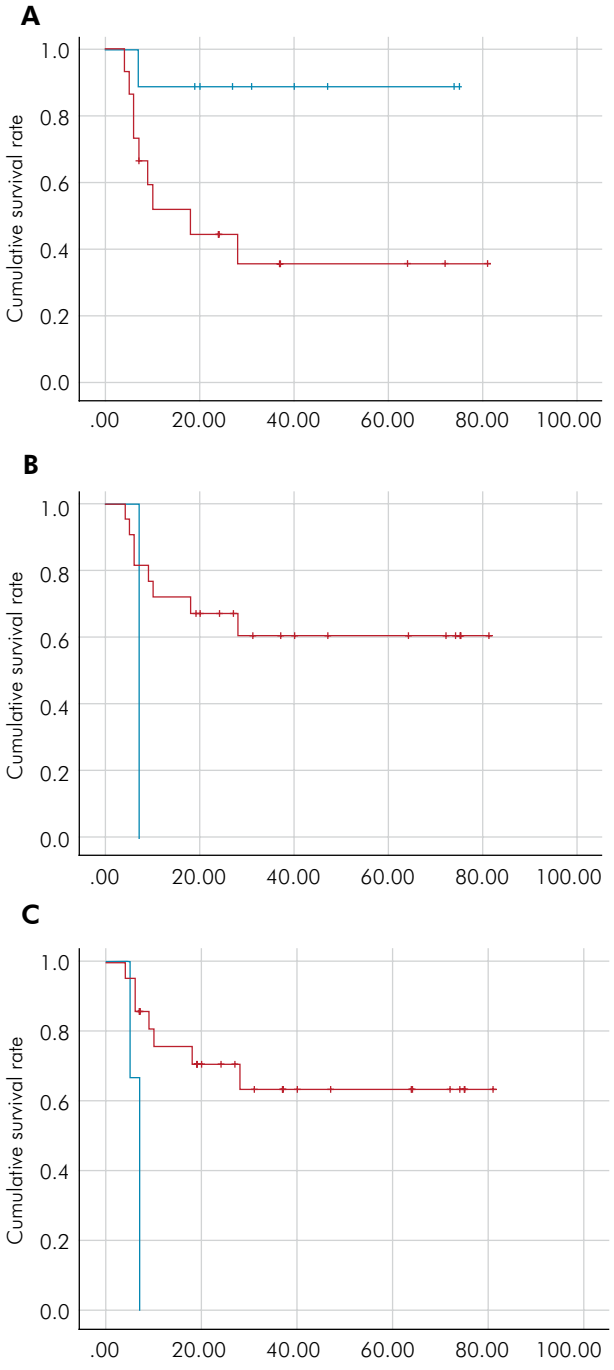

Figure 3. Disease-free survival graphics. A: Graph representing the tumor site. The blue line represents the anterior region. The red line represents the posterior region. B: Graph representing the bone volume variable. The blue line corresponds to cases in which the bone volume was up to 86.47. The red line refers to cases where the bone volume was higher than 86.47. C: Graph representing the bone mineral density variable. The blue line represents cases with bone mineral density up to 1.03. The red line represents cases with a bone mineral density higher than 1.03. All the x axis represents the follow-up, in months.

revealed just a tendency toward a higher risk of 3.6 for the worst OS (p = 0.15).

Discussion

This study focused on patients who underwent mandibulectomy as part of their OSCC treatment. The predominance of male patients aligns with the known prevalence of OSCC in the Brazilian population.[23] The posterior region of the mandible was found to be the most common site of tumor occurrence. The remodeling-associated genes RANKL, RANK, and SOST were mostly downregulated, while OPG presented subexpression in all cases. These findings could suggest a state of partial metabolic inertia in the bone close to the tumor, independent of its invasion status. This observation is supported by the absence of both normal and overexpression conditions in the well-known RANK/RANKL/OPG bone pathway. In addition, regarding other features analyzed, the relationship between bone volume, mineral density, and the location of the tumor within the mandible was found to significantly impact DFS.

The molecular analysis in our study revealed low expression levels for all the remodeling-related genes investigated. Notably, OPG was downregulated in all cases. Although the complete mechanism underlying the reduction in bone metabolic activity is uncertain, it was intriguing to notice that the expected remodeling process in the bone adjacent to the tumor was not observed. Yet, the expression of the 4 genes analyzed in our study did not directly influence survival rates, suggesting a potential link between this metabolic inertia and the surprisingly low levels of gene expression. Another hypothesis is that if the bone does not express these genes, the lesion would probably be the major source of these metabolism-related genes for the bone remodeling needed in the affected area. Immunoexpression of RANKL and RANK in OSCC have already been reported, for both buccal and gingival SCCs, without and with bone invasion, respectively. Strong cytoplasmic staining was found for both molecules, as well as weak to negative cytoplasmic staining for OPG was also reported.[24] Furthermore, the SOST gene, which encodes

sclerostin, showed lower levels in the mandible. Sclerostin, primarily produced by osteocytes, plays a crucial role in regulating the paracrine action of WNT signaling and acts to regulate the activity of osteoblasts and osteoclasts.[13,25]

Microarchitectural analysis of cortical bone in our study revealed that bone volume was a statistically significant influence on local recurrence or metastasis. Altogether, patients with lower bone mineral density also experienced worse outcomes in terms of DFS. It is plausible to hypothesize that both reduced bone volume and lower mineral content would facilitate bone invasion, primarily through the initial events of cortical resorption at the tumor surface. It is important to emphasize that in our study, most cases did not have confirmed bone invasion, but the analyzed bone was in close proximity to the tumor. The normal bone adjacent to the tumor, regardless of tumor status, appears to influence prognosis by exhibiting lower mineral content and a lesser amount of bone matrix. These findings align with our previous result,[15] which demonstrated that tumor invasion into the bone causes a decrease in anisotropy, indicating structural alterations and a lack of hierarchical organization of the bone matrix. These observations may serve as early indicators of bone alterations, even without an evident bone invasion.

Several studies have reported that patients with bone invasion have a higher risk of death and worse outcomes.[7,26] In a systematic review and meta-analysis conducted by Dolens et al.,[26] which examined the histopathological findings in OSCC, an analysis of 172 articles revealed an increased risk for poor survival associated with bone invasion. Although our study did not find statistical significance for bone invasion and OS (likely due to the limited number of cases with confirmed invasion, only 8), a trend was suggested based on the p-value obtained in the Cox regression analysis ($p = 0.1$), indicating a relative risk of 3.6. Bone invasion has previously been linked to worse outcomes, and a systematic review investigating whether mandibular invasion could be an independent prognostic factor in OSCC patients found a relationship between mandibular invasion and OS. However, the review concluded that cortical involvement alone did not decrease OS but only the medullary involvement had such an effect. t[27] Mahajan et al.,[28] in a new proposal for a staging classification, observed that cortical bone erosion may not affect survival and that only medullary invasion affects prognosis. In our study, among the 24 cases, 8 had bone involvement, and only 2 patients had verified complete medullary invasion. Regardless of medullary invasion, we observed that cortex involvement may not influence OS but certainly has an impact on the outcomes in terms of recurrence and disease-free survival.

The main limitation of our study is the small number of patients included in a convenience sample. However, the specific protocol for collecting bone samples for both molecular and microarchitectural analysis requires a significant amount of material, which is only available from mandibulectomy procedures. Consequently, it was necessary to focus on this specific patient group, which represents a limited subset of the entire population of OSCC patients. Additionally, conducting molecular analysis on hard tissue is always challenging, mostly due to the numerous steps required for RNA extraction.

Conclusion

Downregulation of RANK, RANKL, and SOST genes were found in the cortical bone of the mandible in patients diagnosed with OSCC. The OPG gene was downregulated in all cases. DFS rates were influenced by bone properties such as bone volume and mineral density. Furthermore, tumors located close to the posterior mandible were associated with worse outcomes.

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