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Prognostic significance of bone morphogenetic protein 6 (BMP6) expression, clinical and pathological factors in clinically node-negative oral squamous cell carcinoma (OSCC)

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Summary

Bone morphogenetic protein 6 (BMP6) has unique properties regarding structure and function in supporting bone formation during development and adult life. Despite its known role in various malignant tumors, the prognostic significance of BMP6 expression in oral squamous cell carcinoma (OSCC) remains unknown. The aim of the study was to investigate immunohistochemical expression of BMP6 in OSCC in correlation with clinical and pathological parameters, disease recurrence and survival. In addition, we investigated other parameters in order to identify prognosticators of neck metastases and final outcome. The study included 120 patients with clinically T1-3N0 OSCC who were primarily surgically treated between 2003 and 2008. There were 99 (82.5%) male and 21 (17.5%) female patients. The five-year disease-specific survival for the whole cohort was 79.7%. Tumors smaller than 2 cm in diameter showed higher incidence of strong BMP6 expression. No statistical correlation was observed between other clinico-pathological factors and BMP6 expression. Expression of BMP6 was not associated with disease recurrence and survival. BMP6 may not serve as prognosticator of final outcome or recurrence in clinically node-negative OSCC subjects. In multivariate analysis predictors of poorer survival were positive surgical margin, moderate tumor cell differentiation and pathological involvement of levels IV and/or V.

Key words: oral cancer, bone morphogenetic protein 6, occult neck metastases, survival, recurrence.

Introduction

Oral cancer is common worldwide malignancy, accounted for 300,000 cases in 2012 (2.1% of the world total), with two-thirds occurring in men (Ferlay et al., 2015). The region with the highest incidence among both males and females was by far Melanesia (22.9 per 100,000 and 16.0 per 100,000, respectively) (Ferlay et al. 2015). It's associated with great morbidity and mortality rates that have not improved in decades despite early detection and therapeutic advances (Lippman et al., 2005).

Bone morphogenetic proteins (BMPs) are growth and differentiation factors, originally isolated as molecules which *in vivo* stimulate ectopic bone and cartilage formation (Urist et al., 1965; Sampath and Reddi, 1981). The role of the BMP6 protein in the process of bone remodeling and metabolism is well known. BMP6 has a potent function in regulation of mesenchymal stem cell differentiation into osteoblasts, where it serves as a key factor in the bone coupling phenomenon (Yuen et al., 2012). Focusing on BMP6 authors showed that the BMP6 was overexpressed in prostate cancer which was significantly correlated with the appearance of distant metastases (Hamdy et al., 1997, Autzen et al., 1998). Despite its known role in various malignant tumors (Wang et al., 2011, Hu et al., 2016, Lee et al., 2014, Vukicevic and Grgurevic, 2009), the prognostic significance of BMP6 expression in oral squamous cell carcinoma (OSCC) remains unknown. In a recent study, Kejner et al. (Kejner et al., 2013) demonstrated that increased expression of BMP6 was associated with bone invasion in OSCC regardless of tumor size.

The aim of the present study was to investigate the immunohistochemical expression of BMP6 in OSCC and to correlate these values with clinical and pathological parameters, occurence of neck metastases, disease recurrence and survival. We decided to explore BMP6 because it is more potent than other BMP molecules due to high resistance to noggin which

acts as an endogenous BMP antagonist (Song et al., 2010). In addition, we investigated other parameters in order to identify prognosticators of neck metastases and final outcome.

Materials and methods

Study was carried out with the approval of the Ethical Committee of the University of Zagreb School of Medicine, University Hospital Dubrava. The study included 120 consecutive patients with newly diagnosed cT1-T3N0 oral squamous cell carcinoma (OSCC) who were primary surgically treated between 2003 and 2008 at the Department of Maxillofacial Surgery, University Hospital Dubrava, Zagreb, Croatia. Standard surgical treatment included intraoral resection for cT1 tumours and intraoral resectionwith or without elective neck dissection for cT2–T3 tumours. A bilateral lymphadenectomy was performed only in cases where the primary tumours approached the midline. Clinically an N0 neck was defined by its absence of palpable or radiographically suspicious lymph nodes identified by computed tomography (CT) (nodes larger than 1 cm with/without area of central necrosis (central low density or inhomogeneity)). The stage of the disease was based on the currently used international TNM classification from 2002 (Sobin and Wittekind, 2002). Patients with adverse histopathological features (positive margin, perineural invasion, extracapsular spread, multiple positive lymph nodes, stage pT3 or pT4) received postoperative irradiation. With daily fractions of 2 Gy, a prophylactic dose of 50 Gy to clinically undissected neck levels was given, with a boost of 60 Gy to the tumor bed and metastases confined to the lymph node and a boost of 62-66 Gy being given to regions of the neck with ECS and/or close/involved margin. These two features were the indication for addition of chemotherapy to adjuvant irradiation (concurrent chemoradiotherapy). Chemotherapy regimen was: cisplatin 100 mg/m^2 on days 1, 22, and 43. The follow-up protocol consisted of history and physical exam every 3, 6, 8 and 12 months, in the first, second, third, and fourth year of surveillance, respectively. Posttreatment CT (primary and neck) was performed within 1 and 2 years after surgical treatment.

Histologically proven neck metastases, in patients not receiving neck dissection initially, detected during the follow-up period with recurrence at the primary site were not considered occult metastases, because nodal spread may have occurred after primary surgical treatment from local recurrence and therefore these patients were excluded from the analysis.

Immunohistochemical analysis

To evaluate BMP6 expression in OSCC, immunohistochemical analysis was employed. To establish a standard procedure for treating histological slides, several conditions were tested. Untreated slide analysis was performed the same way as treated, excluding the epitope demasking procedure. Demasking of the epitope in the slides was done by heat induced epitope retrieval (HIER) in a citrate buffer (Dako) using a microwave. BMP6 was detected utilizing rabbit polyclonal antibody (AbCam ab-134723), while for the negative control only phosphate-buffered saline (PBS) was used.

For each carcinoma, one representative block encompassing both the central and peripheral portions of the tumor was selected. Five μm thick sections were deparaffinised and rehydrated in the descending series of ethanol with a final 1x phosphate-buffered saline (PBS) incubation, after which the HIER procedure was performed. The untreated slides were incubated in 1x PBS during the HIER procedure. To eliminate endogenous peroxidase activity, the sections were pretreated at room temperature with 3% H_2O_2 in methanol for 10 minutes. Sections were then incubated with the 1× PBS, as a negative control, or primary antibody against BMP6 (ab134723) diluted 1:100 in 1× PBS overnight at +4 ºC in a moist chamber[15]. By testing the BMP6 antibody on a smear of HEK293 cells (positive control) we have shown that the antibody is specific (data not shown). The reaction was detected using Histostain SP kit (Invitrogen) while staining was visualized using AEC chromogen. Slides were counterstained in hematoxylin and mounted using ClearMount (Invitrogen).

The percentage of BMP6-positive tumor cells was assessed semi-quantitatively based on a 4-scale scoring system: 0 = no BMP6 expression, 1 = present up to 10% of tumor, 2 = present in 11-50% of tumor, 3 = present in more than 51% of tumor. The intensity of BMP6 expression was determined as negative (0), weak (1), moderate (2) and strong (3). Immunoreactive scores were calculated by multiplication of the percentage of immunopositive cells and staining intensity and the results were divided into three groups: 0 – negative reaction, I – moderate expression (multiplication 1-3), II – strong expression (multiplication 4, 6 or 9).

Statistical analysis

Follow-up intervals were calculated in months from the date of first treatment to the date of last follow-up or death. To assess the association of BMP6 expression and clinicopathological parameters, a χ^2-test or Fisher's exact test were performed, when appropriate. The prognostic significance of BMP6 and clinicopathological parameters on occurrence of neck metastases was determined using logistic regression analysis. The prognostic significance of BMP6 and clinicopathological parameters on survival was assessed using Cox's proportional hazard regression analysis. Variables proved to be statistically significant in univariate model with respect to survival were included in multivariate analysis. Regression data were presented with regression coefficients/standard error ($\beta \pm SE$) and odds ratio (OR) with 95% confidence intervals (95% CIs). Main outcome measure was disease-specific survival (DSS). A DSS event was defined as a death resulting from OSCC. DSS was calculated using the Kaplan-Meier method, while the log-rank test has been used to test differences between the actuarial curves. All statistical analyses were performed using MedCalc statistical software (MedCalc Statistical Software ver. 13.0.2, MedCalc Software bvba, Ostend, Belgium). P values of < 0.05 were considered statistically significant.

Results

There were 99 (82.5%) male and 21 (17.5%) female patients, with a median age of 59 years (range 35 - 94). Clinicopathological characteristics of the study group are summarized in **Table 1**. Seventy-nine patients underwent intraoral resection, while 41 received intraoral resection in conjunction with elective neck dissection (END). Occult neck metastases were found in 49 (40.8%) patients. Among patients with regional metastases, 19 (46.3%) of them had occult neck disease initially (elective neck dissection group) while 30 (42.3%) patients developed lymph node metastases after treatment of the primary tumor (intraoral resection group). All patients with neck recurrence during the follow-up period underwent therapeutic neck dissection, and 27 (87.1%) received postoperative radio(chemo)therapy based on histopathologic findings. The immunohistochemical reaction for BMP6 showed a positive cytoplasmic reaction in tumor cells in 109 of 120 (90.8%) patients. Moderate BMP6 expression was documented in 79 (65.8%) and strong in 30 (25%) patients, respectively, whereas negative reaction was detected in 11 (9.2%) patients (**Figure 1**).

Patients with tumors smaller than 2 cm showed an increased incidence of strong BMP6 expression. No statistical correlation was observed between other clinico-pathological factors and BMP6 expression **(Table 2)**. Furthermore, expression of BMP6 was not associated with disease recurrence and survival (**Table 3, Figure 2**).

In univariate analysis of BMP6 expression and clinicopathologic factors in relation to neck metastases none of the analysed factors proved to be a prognosticator of occult neck disease **(Table 4)**.

The 5 year DSS for the whole cohort was 79.7% **(Figure 3)**. In the univariate model, survival was correlated with the margin status, tumor cell differentiation and nodal status (presence of regional metastases, ECS and metastatic spread to levels IV and/or V) (**Table 5**). In

multivariate analysis predictors of poorer survival were positive surgical margin, moderate tumor cell differentiation and pathological involvement of levels IV and/or V (**Table 6** and **7**).

During the study, 20 of the 120 patients died (16.7%) of disease recurrence. Follow-up information was available for all patients and ranged from 5 to 120 months (mean 45.6 months). All surviving patients were followed for a minimum of 2 years (mean 57.2 months).

Discussion

Oral cancer represents a significant therapeutic challenge because of its aggressive local and unpredictable regional spread. One of the main reasons for high mortality rates is the largely unpredictable regional metastatic spread and significant deterioration of the survival rate once metastasis to the lymphatic system has occurred.

In recent years, numerous molecular-based assays have been introduced but histopathology remains the gold standard for most diagnostic and therapeutic decisions. Immunohistochemisty is an additional and globally available tool that complements histopathological analysis by detecting genes at the protein level (Oliveira et al., 2011). This is the largest study that examined the prognostic significance of BMP6 in OSCC. In addition, it´s the first study analysing the significance of BMP6 in clinically node-negative settings. In this retrospective investigation, an immunohistochemical reaction for BMP6 showed positive cells in most of the patients, which is in accordance with prior reports (Kejner et al., 2013, Raida et al., 1999).

Expression of BMP6 has been proven to be upregulated in SCC of the oesophagus and is associated with a poor prognosis and dedifferentiation of tumor cells (Raida et al., 1999). Molecular data have shown that BMP6, when found in high levels and in conjunction with noggin and sost in squamous cell carcinoma, can predict cancer progression (Yuen et al., 2012). Importantly this relationship was confirmed in prostate, bladder, and colorectal cancers (Yuen et al., 2012). Furthermore, given the fact that these tumors have high propensity for bone metastases, additional studies showed that prostate cancer promotes osteoblastic activity through BMP-6 and that, in addition to its bone effects, suggest that BMPs promote the ability of the prostate cancer cells to invade the bone microenvironment (Dai et al., 2005). On the contrary, some authors suggest that BMP6 may function as an anti-metastasis factor by a

mechanism involving transcriptional repression of microRNA-21 in breast cancer (Du et al., 2009). In addition, in examining the role of BMP6 in head and neck cancer, increased expression of this protein was found to be associated with increased epidermal growth factor receptor (EGFR) expression in OSCC, a known marker of poor prognosis (Kejner et al., 2012). Furthermore, authors demonstrated that increased BMP6 expression is correlated with local aggressivness characterized by bone invasion regardless of primary tumor size. Same data were confirmed with BMP2, where baseline BMP2 protein expression was found in most head and neck squamous cell carcinomas (98%). A high level of BMP2 protein expression was correlated with an increased incidence of local recurrence (Sand et al., 2014). Similarily, a significant increase in tumor cell invasion in response to recombinant human bone morphogenetic protein-2 (rhBMP-2) in all BMP-2 positive cell lines has been documented (Kokorina et al., 2011) though, we could not confirm this finding. In our study, no statistical correlation was observed between BMP6 expression and clinico-pathological factors. Also, there was no relationship between BMP6 expression and bone involvement, which is in contrast to the findings of only study analysing significance of this protein in oral cancer patients (Lee et al., 2014). Furthermore, expression of BMP6 was not associated with disease recurrence and survival which is in accordance with the previously mentioned study (Lee et al., 2014).

An interesting finding in the evaluation of the expression of BMP6 in cancer tissue was observed during carcinogenesis, especially in the more advanced stages of the disease - that neoplastic cells commonly lose their responsiveness to BMPs either due to the loss of expression of their receptors or to the increased synthesis of BMP inhibitors such as noggin (Hsu et al., 2000, Kim et al., 2000, Kim et al. 2004). Therefore, in advanced tumors BMP6 determination (e.g., in biological fluids) would be a more appropriate method for evaluation of BMP6 as a potential prognostic biomarker (Brkljacic et al., 2013). On the contrary, other

BMP members (BMP2 and -4) show high expression in advanced tumors (metastatic disease) compared to non-metastatic OSCC (Soares et al., 2010).

In our study, no factor has been identified as a prognosticator of occult nodal disease. This could be explained by the complexity of the metastatic processes, in which conventional clinical and pathological parameter alone fail to predict lymphatic spread of tumor.

On the contrary, survival was correlated with the margin status, tumor cell differentiation, and nodal status. Additionally, all neck dissection specimen characteristics (positive neck status, extracapsular spread, ≥3 affected lymph nodes, involvement of regions IV and/or V) proved to be significant parameters associated with survival in univariate analysis which emphasis a nodal status and it's features as most important prognosticators of survival of these patients irrespective of other clinical or histopathological parameters. We also carried out a multivariate analysis of factors previously found as important prognosticators of final outcome in the univariate model, which identified positive surgical margin, moderate tumor cell differentiation and pathologic involvement of levels IV, and/or V as predictors of poorer survival.

This study has all the limitations associated with retrospective design. Another weakness of this analysis is the difference in criteria of immunohistochemical biomarker assessement and the fact that study deals with expression of one molecule assessed by immunohistochemistry. There is also heterogeneity in the samples among the studies, which can lead to different results in similar clinical scenarios.

Also, weakness of the study is the managment of the clinically N0 neck. In our study there was a high variation of N0 neck treatment emphasizing the necessity for more evidence based approach and uniform clinical practice. More than half (61%) of the patients with T2 tumors were not submitted to END which is possible explanation for high regional recurrence rate of

42.3% among 'wait and see' subgroup of patients. Although, most of the patients experiencing neck metastases were regionally free from the disease after salvage surgery and (chemo)radiotherapy, nowadays, standard of care for patients with early-stage oral cancer represent elective lymphadenectomy at the time of surgery for primary tumor.

On the other hand, we believe that this data provides insight into the role of BMP6 in tumor tissue determined using immunohistochemistry in tumor progression, metastasis, recurrence and survival of patients with OSCC. Also, study identified other factors which are predictive for final outcome of OSCC subjects in clinically node-negative settings.

In conclusion, expression of BMP6 may not serve as a prognosticator in OSCC. Independent predictors of survival were positive surgical margin, moderate tumor cell differentiation, and pathologic involvement of nodal levels IV and/or V.

Further investigations are needed in order to identify patients at high risk for occult neck disease and to provide new and effective therapeutic strategies.

Acknowledgements and conflict of interest statement

This work was supported by the Ministry of Science, Education and Sport, Republic of Croatia. We disclose any other commercial associations, current and within the past five years, that might pose a potential, perceived or real conflict of interest. These include grants, patent licensing arrangements, consultancies, stock or other equity ownership, donations, advisory board memberships, or payments for conducting or publicizing the study.

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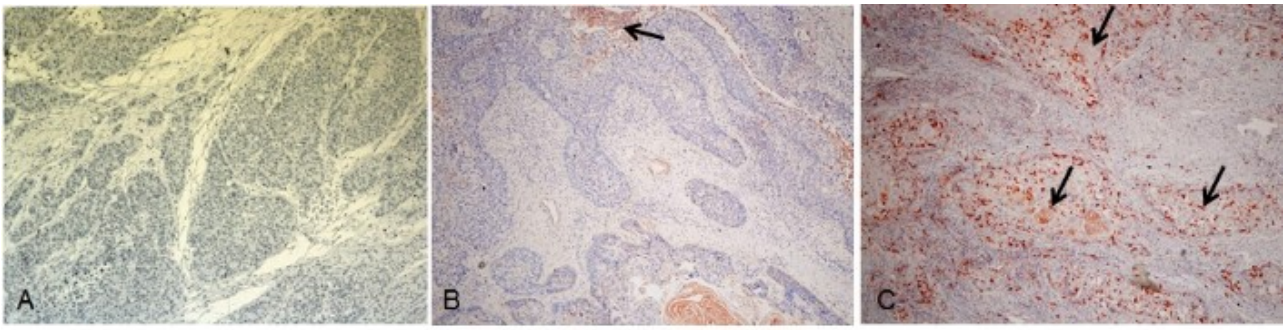
Captions to illustrations

Figure 1. Immunohistochemical reaction in OSCC tumour cells A. Negative reaction; B. weak BMP6 reaction in keratin and only a minority of the tumor cells (arrow); C. strong positive BMP reaction in the majority of tumor cells (arrows).

Figure 2. Five-year DSS of the patients with negative, moderate and strong BMP6 expression. There is no statistical difference between survival curves (P = 0.793).

Figure 3. Five-year DSS of the study group.


A
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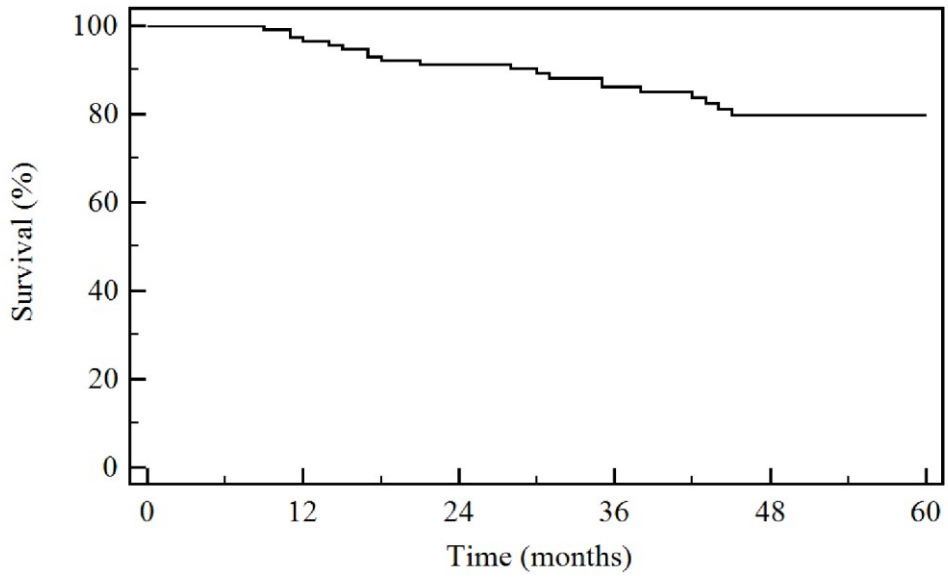
100
80
60
40
20
0
Survival (%)
0
12
24
36
48
60
Time (months)

Figure 3.

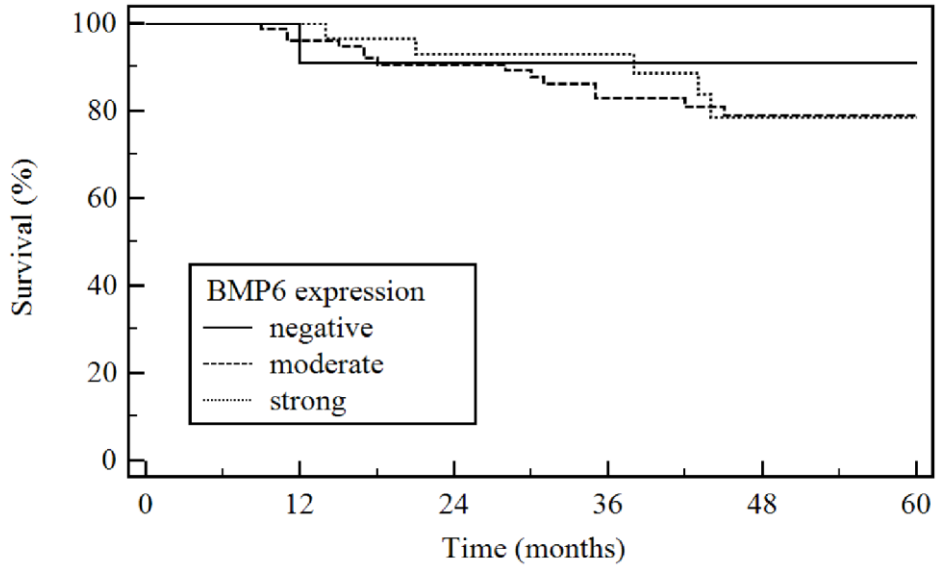
100
80
60
40
20
0
Survival (%)
0
12
24
36
48
60
Time (months)
BMP6 expression
negative
moderate
strong

Table 1. Clinical and histopathological characteristics of the cohort.

Characteristics	Number of patients (%)
Age (years)	
≤ 59	63 (52.5)
> 59	57 (47.5)
Gender	
Male	99 (82.5)
Female	21 (17.5)
Primary site	
Floor of the mouth	56 (46.7)
Tongue	38 (31.7)
Retromolar space	15 (12.5)
Lower gingiva	11 (9.1)
T classification	
T1	35 (29.2)
T2	72 (60.0)
T3	13 (10.8)
Tumor thickness	
≤ 0.8 cm	64 (53.3)
> 0.8 cm	56 (46.7)
Margin status	
Negative	116 (96.7)
Positive	4 (3.3)
Differentiation	
Well	54 (45.0)
Moderate	59 (49.2)
Poor	7 (5.8)
Perineural invasion	
No	62 (51.7)
Yes	58 (48.3)
Extracapsular spread	
No	16 (32.7)
Yes	33 (67.3)
Adjuvant RT	
No	72 (60.0)
Yes	48 (40.0)
Survival	
NED†	100 (83.3)
DOD‡	20 (16.7)

†no evidence of disease; patients who died from other causes were censored at date of death

‡ died of disease

Table 2. Association of BMP6 expression and clinical and histopathological factors.

Parameter	Groups	BMP6			P value*
		negative	moderate	strong	
Clinical data					
Age, years	≤ 59	6 (5.0)	39 (32.5)	18 (15.0)	0.402
	> 59	5 (4.2)	40 (33.3)	12 (10.0)	
Gender	men	9 (7.5)	65 (54.2)	25 (20.8)	1.00
	women	2 (1.7)	14 (11.7)	5 (4.2)	
Tumor subsite	sublingual	7 (5.8)	32 (26.7)	17 (14.2)	0.338
	tongue	2 (1.7)	30 (25.0)	6 (5.0)	
	retromolar space	1 (0.8)	9 (7.5)	5 (4.2)	
	mandible gingiva	1 (0.8)	8 (6.7)	2 (1.7)	
Tumor diameter, cm	≤ 2,0	4 (3.3)	20 (16.7)	10 (8.3)	0.585
	> 2,0	7 (5.8)	59 (49.2)	20 (16.6)	
Histopathological data					
Tumor diameter, cm	≤ 2,0	6 (5.0)	32 (26.7)	21 (17.5)	**0.021**
	> 2,0	5 (4.2)	47 (39.2)	9 (7.5)	
Margin status	negative	10 (8.3)	76 (63.3)	30 (25.0)	0.571
	positive	1 (0.8)	3 (2.5)	0 (0)	
Periostal invasion	no	5 (8.9)	19 (33.9)	11 (19.6)	0.209[a]
	yes	0 (0)	18 (32.1)	3 (5.3)	
Bone invasion	no	5 (8.9)	26 (46.4)	12 (21.4)	0.480[a]
	yes	0 (0)	11 (19.6)	2 (3.6)	
Differentiation	well	4 (3.3)	39 (32.5)	11 (9.2)	0.381
	moderate	5 (4.2)	38 (31.7)	16 (13.3)	
	poor	2 (1.7)	2 (1.7)	3 (2.5)	
Perineural invasion	no	8 (6.7)	39 (32.5)	15 (12.5)	0.837
	yes	3 (2.5)	40 (33.3)	15 (12.5)	
Regional metastases	no	8 (6.7)	45 (37.5)	18 (15.0)	1.00
	yes	3 (2.5)	34 (28.3)	12 (10.0)	
Number of involved lymph nodes	≤ 3	2 (4.1)	25 (51.0)	7 (14.3)	0.473[b,c]
	> 3	1 (2.0)	9 (18.4)	5 (10.2)	
Lowest involved region	≤ 3	3 (6.1)	25 (51.0)	8 (16.3)	0.708[c,d]
	> 3	0 (0)	9 (18.4)	4 (8.2)	
Extracapsular spread	no	1 (2.0)	11 (22.4)	4 (8.2)	1.00[c]
	yes	2 (4.1)	23 (47.0)	8 (16.3)	

* negative and moderate BMP6 expression was compared with strong BMP6 imunoreactivity
[a] from 56 resected mandibles
[b] comparison of up to 3 involved lymph nodes to 4 or more
[c] from 49 patients with regional metastases
[d] comparison of level 1, 2 or 3 involvment with involvment of level 4 or 5

Table 3. Association of BMP6 expression and disease recurrence.

Parameter	Groups	Local recurrence N (%)		Regional metastases N (%)	
		No	**Yes**	**No**	**Yes**
BMP6	negative	10 (9.3)	1 (8.3)	8 (11.3)	3 (6.1)
	moderate	76 (65.7)	3 (66.7)	45 (63.4)	34 (69.4)
	high	29 (25.0)	1 (25.0)	18 (25.3)	12 (24.5)
P value		0,688		0,605	

Table 4. Association between clinical/histopathological parameters and regional metastases.

Parameter	Groups	Regional metastases N (%)		P value
		No	Yes	
Clinical data				
Age, years	≤ 59	35 (49.3)	28 (57.1)	0,397
	> 59	36 (50.7)	21 (42.9)	
Gender	men	58 (81.7)	41 (83.7)	0,778
	women	13 (18.3)	8 (16.3)	
Tumor subsite	sublingual	35 (49.3)	21 (42.9)	
	tongue	19 (26.8)	19 (38.8)	0,231[a]
	retromolar space	8 (11.3)	7 (14.3)	0,520[a]
	mandible gingiva	9 (12.7)	2 (4.1)	0,231[a]
Tumor diameter, cm	≤ 2,0	19 (15.8)	15 (12.5)	0,683
	> 2,0	52 (43.3)	34 (29.3)	
Histopathological data				
Tumor diameter, cm	≤ 2,0	33 (27.5)	26 (21.7)	0,578
	> 2,0	38 (31.7)	23 (19.1)	
Tumor thickness, cm	≤ 0,8	42 (59.2)	22 (44.9)	0,124
	> 0,8	29 (40.8)	27 (55.1)	
Margin status	negative	68 (95.8)	48 (98.0)	0,500
	positive	3 (4.2)	1 (2.0)	
Periostal infiltration	no	20 (60.6)	15 (65.2)	0,725[b]
	yes	13 (39.4)	8 (34.8)	
Bone infiltration	no	23 (69.7)	20 (87.0)	0,122[b]
	yes	10 (30.3)	3 (13.0)	
Differentiation	well	37 (52.1)	17 (34.7)	
	moderate	30 (42.3)	29 (59.2)	0,061[a]
	poor	4 (5.6)	3 (6.1)	0,549[a]
Perineural invasion	no	38 (53.5)	24 (49.0)	0,625
	yes	33 (46.5)	25 (51.0)	
Immunohistochemistry				
BMP6 expression	negative	8 (11.3)	3 (6.1)	
	moderate	45 (63.4)	34 (69.4)	0,327[a]
	strong	18 (25.3)	12 (24.5)	0,457[a]
Treatment data				
Mandible resection	no	38 (53.5)	26 (53.0)	
	marginal	23 (32.4)	14 (28.6)	0,783[a]
	segmental	10 (14.1)	9 (18.4)	0,602[a]

[a] compared with first subgroup of patients
[b] from 56 resected mandibles

Table 5. Univariate analysis of 5-year DSS survival.

Parameter	Groups	N	5-year DSS ± SE%	P value
Clinical data				
Age	≤ 59	63	79.8 ± 5.9	0.710
	> 59	57	79.5 ± 5.9	
Gender	men	99	81.7 ± 4.4	0.373
	women	21	70.0 ± 11.8	
Tumor subsite	sublingual	56	76.2 ± 6.4	0.636
	tongue	38	80.9 ± 7.2	
	retromolar space	15	92.3 ± 7.4	
	mandible gingiva	11	75.8 ± 15.6	
Tumor diameter,cm	≤ 2,0	34	86.9 ± 6.1	0.417
	> 2,0	86	79.2 ± 4.7	
Histopathological data				
Tumor diameter, cm	≤ 2,0	59	81.6 ± 5.3	0.935
	> 2,0	61	81.2 ± 5.4	
Margin status	negative	116	82.0 ± 4.1	**< 0.001**
	positive	4	0 ± 0	
Tumor thickness, cm	≤ 0,8	64	82.3 ± 5.2	0.479
	> 0,8	56	76.2 ± 6.9	
Periost invasion	no	35	79.0 ± 7.8	0.474[a]
	yes	21	63.3 ± 13.6	
Bone invasion	no	43	75.4 ± 7.7	0.789[a]
	yes	13	68.2 ± 15.8	
Differentiation	well	54	89.4 ± 4.5	**0.035**
	moderate	59	68.5 ± 7.0	
	poor	7	–	
Perineural invasion	no	62	85.0 ± 5.0	0.253
	yes	58	73.7 ± 6.7	
Immunohistochemistry				
BMP6 expression	negative	11	90.9 ± 8.7	0.793
	moderate	79	78.9 ± 5.1	
	strong	30	78.4 ± 8.8	
Neck dissection specimen				
Regional metastases	no	71	88.9 ± 4,0	**0.025**
	yes	49	68.2 ± 7,4	
Number of involved nodes	≤ 3	34	77.9 ± 8,0	**0.023[b,c]**
	> 3	15	46.6 ± 14,4	
Lowest involved region	≤ 3	36	83.2 ± 7,0	**<0.001[c,d]**
	> 3	13	28.1 ± 13,7	
Extracapsular spread	no	16	90.9 ± 8,7	**0.036[c]**
	yes	33	58.1 ± 9,5	

[a] from 56 resected mandibles
[b] comparison of up to 3 involved lymph nodes to 4 or more
[c] from 49 patients with regional metastases
[d] comparison of level 1, 2 or 3 involvment with involvment of level 4 or 5

Table 6. Multivariate Cox's proportional hazard regression analysis.

Parameter	Group	N	Survival, statistics[b]		
			β ± SE	**P value**	**OR (95% CI)**
Extracapsular spread	no	16			
	yes	33	1.60 ± 1.07	0.133	4.97 (0.62 – 39.76)
Number of involved lymph nodes	≤ 3	34			
	> 3	15	0.86 ± 0.57	0.133	2.36 (0.77 – 7.21)
Lowest involved region	≤ 3	36			
	> 3	13	1.81 ± 0.57	**0.002**	6.13 (2.0 – 18.79)

[b]calculation based on 49 patients with regional metastases

Table 7. Multivariate Cox's proportional hazard regression analysis.

Parameter	Group	N	Survival, statistics		
			β ± SE	**P value**	**OR (95% CI)**
Margin status	negative	116			
	positive	4	2.84 ± 0.68	**<0.001**	17.16 (4.53 – 64.97)
Differentiation	well	54			
	moderate	59	1.24 ± 0.52	**0.017**	3.46 (1.26 – 9.52)
	poor	7	-[a]	0.964	-[a]
Regional metastases	no	71			
	yes	49	0.85 ± 0.48	0.076	2.33 (0.92 – 5.93)

[a] not calculated due to high SE