Oral Oncology 51 (2015) 247–253

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Upregulation of osteoprotegerin expression correlates with bone invasion and predicts poor clinical outcome in oral cancer

G. Russmueller [a], D. Moser [a], T. Würger [b], F. Wrba [b], P. Christopoulos [c], G. Kostakis [c], R. Seemann [a], V. Stadler [a], G. Wimmer [a], G. Kornek [d], A. Psyrri [e], M. Filipits [f], C. Perisanidis [a,*]

[a] Department of Cranio-, Maxillofacial and Oral Surgery, Medical University of Vienna, Austria
[b] Department of Clinical Pathology, Medical University of Vienna, Austria
[c] Department of Maxillofacial and Oral Surgery, University of Athens, Greece
[d] Department of Medicine I, Medical University of Vienna, Austria
[e] Division of Oncology, Second Department of Internal Medicine, Attikon University Hospital, Athens, Greece
[f] Department of Medicine I, Institute of Cancer Research, Medical University of Vienna, Austria

ARTICLE INFO

Article history:
Received 8 September 2014
Received in revised form 10 November 2014
Accepted 15 November 2014
Available online 17 December 2014

Keywords:
Oral cancer
Osteoprotegerin
Receptor activator of nuclear factor-kappa B
RANK ligand
Prognosis
Neoadjuvant therapy

SUMMARY

Objectives: We aimed to determine the prognostic significance of receptor activator of nuclear factor kappa-B ligand (RANKL), RANK and osteoprotegerin (OPG) in patients with oral squamous cell carcinoma (OSCC).

Materials and methods: The protein expression of RANKL, RANK and OPG was assessed by immunohistochemistry on pretreatment biopsies of 93 patients with locally advanced OSCC who received preoperative chemoradiotherapy (CRT). The primary endpoint was cancer-specific survival. Secondary endpoints were correlation of biomarkers with bone invasion and pathological tumor response. Kaplan–Meier curves and Cox regression models were used for survival analyses.

Results: A significantly higher OPG expression was demonstrated in patients with malignant bone invasion and non-responders to CRT as compared to patients without bone invasion and responders ($p = 0.032$ and $p = 0.033$, respectively). Multivariate analysis revealed that higher OPG expression was independently associated with shorter cancer-specific survival ($p = 0.04$). The expression status of RANKL and RANK was not significantly related to clinicopathological characteristics and had no impact on survival of OSCC patients.

Conclusion: Upregulation of OPG expression is associated with bone invasion, poor pathological tumor regression to neoadjuvant CRT, and worse long-term cancer-specific survival in patients with locally advanced OSCC. Our results indicate that OPG may be a novel prognostic biomarker in oral cancer.

Introduction

Despite significant treatment advances, the overall survival of patients with oral squamous cell carcinoma (OSCC) continues to hover around 50% at 5 years, mainly because patients frequently develop locoregional recurrence and/or metastatic disease [1]. Non-responding patients not only have a higher mortality rate, but also suffer adverse effects from the treatment itself. Thus, novel cancer biomarkers that have the potential to predict tumor response are urgently needed to establish individualization of cancer therapy and improve patient survival.

The receptor activator of nuclear factor kappa-B ligand (RANKL), its cognate receptor RANK and osteoprotegerin (OPG) belong to the tumor necrosis factor (TNF) superfamily and play a pivotal role in the regulation of bone remodeling [2]. RANKL, which is expressed on the surface of osteoblasts, stromal cell and T lymphocytes, binds to RANK on the surface of osteoclast precursors and mature osteoclasts [3]. The interaction between RANKL and RANK activates an intracellular signaling cascade that leads to differentiation and survival of osteoclasts [4]. OPG, released by osteoblasts and stromal cells, functions as a soluble decoy receptor for RANKL preventing it from binding to and activating RANK [5].

There are multiple lines of evidence indicating that abnormal expression of the RANKL/RANK/OPG molecular triad mediates tumor progression and metastasis in a wide range of malignancies, including breast, prostate, colorectal, lung, bladder and gastric

* Corresponding author at: Medical University of Vienna, Department of Cranio-, Maxillofacial and Oral Surgery, Waehringer Guertel 18-20, 1090 Vienna, Austria. Tel.: +43 1 40400 56690; fax: +43 1 40400 42530.
E-mail address: christos.perisanidis@meduniwien.ac.at (C. Perisanidis).

http://dx.doi.org/10.1016/j.oraloncology.2014.11.010
1368-8375/

cancers [6–11]. It has been demonstrated that the RANKL/RANK/OPG system plays a critical role in the crosstalk between cancer cells and the bone microenvironment, promoting a vicious cycle between bone resorption and tumor growth in bone [12]. Specifically, cancer cells induce osteoclastic bone resorption by producing RANKL as well as other osteolytic factors, such as the parathyroid hormone-related protein (PTHrP), which then stimulate the production of RANKL and inhibit the production of OPG in osteoblasts and bone marrow stromal cells [13]. The increased osteoclast-mediated bone resorption releases growth factors from the bone matrix, particularly the tumor growth factor β (TGF-β), that stimulate tumor growth [14].

In cancer cells, activation of RANKL/RANK signaling induces epithelial-mesenchymal transition (EMT), which is characterized by the loss of E-cadherin-induced cell–cell contacts and apical–basal polarization, as well as increased cell motility, thus mediates tumor cell migration, invasion and metastasis [15,16]. Evidence indicates that OPG has the ability to bind to and neutralize the apoptotic function of TNF-related apoptosis-inducing ligand (TRAIL) [17]. OPG-mediated resistance to TRAIL-induced apoptosis provides cancer cells with a critical growth advantage, contributes to tumor progression, and correlates with tumor aggressiveness and poor patient prognosis [18]. In OSCC cells, RANKL, RANK and OPG expression has been detected in vitro and in vivo studies [19–22]. Recent reports have demonstrated the crucial role of the RANKL/RANK/OPG triad in osteoclast-mediated bone destruction associated with OSCCs, which frequently invade the mandible or the maxilla [23–25]. However, to date only a limited number of studies have assessed the prognostic significance of the RANKL/RANK/OPG system in oral cancer [26,27]. Accordingly, the present study aimed to determine whether RANKL, RANK and OPG immunohistochemical (IHC) expression in pretreatment biopsies could predict pathological tumor response, bone invasion and cancer-specific survival (CSS) in patients with locally advanced OSCC who received preoperative chemoradiotherapy (CRT).

Materials and methods

Study population and treatment

The local Institutional Review Board approved this retrospective cohort study. The study population comprised patients with primary squamous cell carcinomas of the oral cavity who were treated with curative-intent neoadjuvant CRT followed by radical cancer surgery at the Departments of Radiotherapy and Cranio-Maxillofacial and Oral Surgery, at the Medical University of Vienna, between 2000 and 2009. Inclusion criteria were previously untreated primary OSCC, availability of pretreatment tumor biopsy, tumor node metastasis (TNM) stages III and IV, World Health Organization (WHO) performance status 0–2 with adequate laboratory parameters, and clear resection margins (R0). Exclusion criteria were presentation with distant metastatic disease (M1), and previous history of squamous cell carcinomas of the head and neck. All patients received neoadjuvant CRT consisting of mitomycin C (15 mg/m^2, i.v. bolus injection on day 1) administered with 5-fluorouracil (750 mg/m^2/day, continuous infusions on days 1–5) and concurrent radiotherapy over 5 weeks up to a total dose of 50 Gy (25 fractions of 2 Gy per day). Surgery was performed 4–8 weeks after the completion of radiation therapy. Patients were followed up on for a minimum of 5 years or until death. The study complied with reporting recommendations for tumor marker prognostic studies (REMARK) criteria [28].

For all eligible patients, demographic, clinicopathological and follow-up data were extracted from the Vienna General Hospital Patient Information System (AKIM). The clinical and pathological tumor stages were reported according to the TNM classification of the International Union Against Cancer (UICC) [29]. The WHO classification was used to report the histological tumor type and grading. Pathological response to neoadjuvant CRT was assessed in surgical specimens as previously described [30], according to vitality of residual tumor cells in relation to the tumor bed: no vital tumor cells, <5% of vital tumor cells, 5–50% of vital tumor cells and more than 50% of vital tumor cells [regression grades (RG) 1, 2, 3 and 4, respectively]. Regression grades were grouped as responder (RG1/RG2) and non-responder (RG3/RG4).

Immunohistochemical analysis and evaluation of staining

To study the expression RANKL, RANK and OPG, formalin-fixed, paraffin-embedded biopsy specimens archived at the Department of Pathology at the Medical University of Vienna, were subjected to standard IHC analysis. In brief, representative tumor specimens were sectioned into blocks (4 μm thickness). One section was stained with haematoxylin and eosin to verify the presence of vital carcinoma cells and adjacent sections were used for immunostaining. Sections were deparaffinized in xylene and rehydrated in a decreasing ethanol series (100–70%) and water. For antigen retrieval, slides were submerged in proteinase K for 15 min at 37 °C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase prior to incubation with primary antibodies. To prevent nonspecific binding sections were blocked with serum from the species that the secondary antibody was generated in (Horse serum Gibco® 26050–070; Goat serum PAA B11–035). Sections were then incubated with antibodies specific for RANKL (clone FL-317; Santa Cruz Biotechnology, Santa Cruz, CA), RANK (clone C-20; Santa Cruz Biotechnology, Santa Cruz, CA), and OPG (Clone 69127; R&D Systems, Minneapolis, MN) (1:100 each) overnight at 4 °C. To remove antibody solution tissue sections were washed in PBS three times and then incubated for 30 min at room temperature with biotinylated secondary antibody (Anti-Goat IgG Vectastain BA-9500; Anti-Rabbit IgG Vectastain BA-1000; Anti-Mouse IgG Vectastain BA-2000). Following this, sections were washed twice with PBS and then incubated with avidin–biotin complex conjugated to horseradish peroxidase. Positive immunohistochemical reactions were revealed using the avidin–biotin-peroxidase complex method (ABC) (Vectastain Elite ABC Kit, Vectastain PK6100). Peroxidase binding was visualized by DAB chromogenic staining (3,3′Diaminobenzidine DAB Fluka 32750).

An experienced pathologist, blinded to patient clinical data and outcome, evaluated IHC staining by light microscopy. For assessment of RANKL, RANK and OPG expression, a semiquantitative scoring method, based on staining intensity and percentage of positive cells to create an IHC score, was used as previously described [31,32]. For each slide a IHC score was calculated on a continuous scale of 0–300 using the following formula: 1 × (percentage of cells that stained weakly [1+]) + 2 × (percentage of cells that stained moderately [2+]) + 3 × (percentage of cells that stained strongly [3+]). Staining intensity was graded into 4 categories: no staining (0), weak (1+, light brown staining visible only at high magnification), intermediate (2+, between 1+ and 3+), and strong (3+, visible at low magnification, dark-brown linear staining). The percentages of positive cells showing the different staining intensities were scored visually.

Statistical methods

The primary endpoint of this study was cancer-specific survival (CSS) defined as the time from surgery to death from OSCC or last follow-up. Secondary objectives were to determine whether the IHC expression of RANKL, RANK, and OPG in pretreatment biopsies could predict pathological response to neoadjuvant CRT and bone