doi: 10.1111/j.1600-0714.2009.00793.x

J Oral Pathol Med (2009) 38: 753–758 © John Wiley & Sons A/S · All rights reserved

interscience.wiley.com/journal/jop

# Immunohistochemical expression of RANKL, RANK, and OPG in human oral squamous cell carcinoma

Fu-Hsiung Chuang<sup>1,2</sup>\*, Shue-Sang Hsue<sup>3</sup>\*, Chung-Wei Wu<sup>4</sup>, Yuk-Kwan Chen<sup>5,6</sup>

<sup>1</sup>Department of Endodontics & Operative Dentistry, Faculty of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; <sup>2</sup>Division of Dentistry, Department of Endodontics & Operative Dentistry, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan; <sup>3</sup>School of Dentistry, China Medical University, Taichung, Taiwan; <sup>4</sup>Division of Dentistry, Department of Oral & Maxillofacial Surgery, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan; <sup>5</sup>Department of Oral Pathology, Faculty of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; <sup>6</sup>Division of Dentistry, Department of Oral Pathology, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan;

BACKGROUND: The mechanism of oral squamous cell carcinoma (SCC) invading jawbone remains controversial. Interactions between receptor activator of NF- $\kappa$ B (RANK) and its ligand (RANKL) are required for osteoclastogenesis. The binding of RANK and RANKL induces differentiation of osteoclasts, leading to bony destruction. Osteoprotegerin (OPG), a decoy receptor for RANKL, also binds to RANKL by competing with RANK, and this could protect against osseous destruction.

MATERIALS AND METHODS: Immunoexpression of RANKL, RANK, and OPG in 25 cases of human buccal SCCs without bony invasion and 15 cases of gingival SCCs with mandibular bony invasion was investigated. Normal oral mucosa from five individuals without betel-quid chewing or cigarette smoking was used as a control. The scores are designated as percentage of positive staining  $\times$  intensity of staining for each section.

RESULTS: Strong cytoplasmic staining of RANKL proteins is detected in cancer cells of both buccal and gingival SCCs. The same protein is identified in cytoplasm of osteoclasts for all cases involving bony invasion. Strong cytoplasmic staining of RANKL is confined to basal layer for all normal mucosa. A similar staining pattern is noted for RANK protein in all buccal and gingival SCCs. An absence of staining of RANK protein is noted for all normal tissues. Weak to negative cytoplasmic stained OPG protein is present in all buccal and gingival SCCs, but is absent in all normal tissues.

CONCLUSION: These findings suggest the potential value of the RANK/RANKL/OPG pathway as biomarkers in human oral SCCs.

| Oral Pathol Med (2009) 38: 753-758

**Keywords:** OPG; oral squamous cell carcinoma; osteoclastogenesis; RANK; RANKL

## Introduction

Oral squamous cell carcinoma (SCC) is a common malignant tumor of the oral and maxillofacial region (1), especially in South-East Asian countries (2). Oral SCC may invade the jawbone and this usually requires surgical resection, which potentially leads to compromised jaw function.

Although there have been some breakthroughs (3, 4), the precise mechanism by which oral SCC invades bone remains incompletely described. It is claimed that oral SCC invades bone by direct extension and not by metastasis. Although some controversy still exists, the bony destruction associated with such invasion is thought to be mediated by osteoclasts rather than directly by the carcinoma itself (3, 4). Osteoclasts are multinucleated cells that are responsible for bone resorption. A previous study has reported that osteoclasts are more common in bone invaded by cancer than in bone that is not (5).

Three proteins have been shown to potently stimulate osteoclastogenesis, namely, receptor activator of NF- $\kappa$ B ligand (RANKL) (6), osteoprotegerin (OPG) ligand (OPGL) (7), and tumor necrosis factor (TNF)-related activation-induced cytokine (TRANCE) (8). RANK (a member of the TNF receptor family) is the cognate receptor of RANKL and is expressed in high levels on osteoclast precursors (9). Interactions between RANKL and RANK have been shown to induce differentiation from pre-osteoclasts to osteoclasts, causing bone destruction (10). OPG is a decoy receptor for RANKL (11) and can therefore compete with RANK, which could protect against bone destruction (12, 13). Osteoclastogenesis is consequently modulated through a balance between OPG and RANKL (14).

Correspondence: Dr Yuk-Kwan Chen, Department of Oral Pathology, School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung Medical University Hospital, 100 Shih Chuan 1st Road, Kaohsiung, Taiwan. Tel: +886 7 3121101(ext. 2755), Fax: +886 7 3210637, E-mail: k0285@ms22.hinet.net \*These authors contributed equally to this paper. Accepted for publication April 15, 2009

Nagai et al. (15) were the first to report on the expression of RANKL in oral SCC cells. Subsequently, Tada et al. (16) have suggested that oral SCC cells can induce suppression of OPG, thus promoting osteoclastogenesis. These two reports were both chiefly *in vitro* studies of human oral cancer cell lines. It remains to be elucidated *in vivo* whether RANKL, RANK, and OPG proteins are also expressed in human SCC of the oral cavity. This study looks for immunohistochemical evidence of the expression of these three proteins in surgical samples of human buccal SCC without bony invasion and also gingival SCC with such invasion.

## Materials and methods

### Study population

Surgical specimens of the SCC in the oral cavity used in this study were obtained from 40 patients (36 males and four females) after approval from our institution. These patients were aged between 47 and 68 years (mean age 55.6 years), and had undergone curative resection in the Oral & Maxillofacial Surgery Department of our institution. The disease stage was defined as stage I-IV in accordance with the Tissue, Nodes, Metastases (TNM) classification (17). Out of the total 40 samples we collected, there were 25 buccal SCCs with no mandibular invasion (stage I: six cases; stage II, nine cases; stage III: 10 cases), and 15 gingival SCCs with mandibular invasion (stage IV: 15 cases; Fig. 1). None of the patients received any pre-operative radiotherapy or chemotherapy. Most identified malignancies were welldifferentiated, except for four that were deemed to be moderately differentiated. Normal oral mucosa was taken from five healthy individuals between 36 and 62 years (mean age: 47 years), under approval from our institution, and used as control. They denied betel-quid chewing or cigarette smoking. The resected oral tissue was fixed in 10% neutral buffered formalin solution for approximately 24 h, dehydrated in graded alcohols,

cleared in xylene, and then embedded in paraffin for immunohistochemical examination.

#### Immunohistochemistry

Staining was performed using the standard avidin-biotin peroxidase complex (ABC) method (18). Rabbit polyclonal antibodies against human RANKL, RANK, and OPG (Cat. no: ab9957, ab22106, and ab9986, respectively; Abchem Corporation, Cambridge, UK) were used. Tissue sections were mounted on gelatin-chrome alum-coated slides. Following repeated deparaffinization in xylene and rehydration in a decreasing-concentration ethanol series (absolute, 95%, 70%, and 30%) ethanol, and then water), tissue sections were microwave-treated thrice (5 min each) in a citrate buffer (10 mM; pH = 6.0) to retrieve antigenicity. Endogenous peroxidase activity was blocked by soaking the sample in 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in methanol for 60 min. Prior to staining, a 10% solution of normal rabbit serum was applied for 60 min to tissue sections to inhibit non-specific staining. These sections were subsequently incubated with antibodies against RANKL, RANK, and OPG (1:100, each) overnight at 4°C. Following subsequent rinsing with Tris-buffered saline (TBS, three times, 10 min each), tissue sections were then incubated for 60 min at room temperature with biotin-conjugated goat anti-rabbit IgG (1:100; Vector, Burlingame, CA, USA). Following this, all sections were washed with TBS again (three times, 10 min each) and then incubated with avidin-biotin complex conjugated to horseradish peroxidase (Dako, Santa Barbara, CA, USA) for a further 60 min. After washing with TBS (three times, 10 min each), peroxidase binding was visualized as brown reaction products via a benzidine reaction. The scores of percentage of positive immunostaining (P) were classified as: 0 (<1%); 1 (1–24%); 2 (25–49%); 3 (50–74%); and 4 (75–100%), whereas the scores for intensity of staining (I) were classified as 0, no staining; 1, light yellow color (weak staining); 2, brown color (moderate strong staining); and 3, dark brown color (strong staining). The total scores (S) are designated as  $P \times I$  for each section (19). Statistical analysis was performed according to the method of Mann and Whitney using the JUMP 6.0 software (SAS Inst. Inc., Cary, NC, USA). P values less than 0.05 were considered significant. An adequate internal positive control in each set of experiments ensured the reproducibility of the staining process. A negative control, in which the primary antibody step was omitted, was also included in each set of experiments.

To enumerate the positive-stained cells, 300 cells were examined in at least five areas (slides were divided into nine equal areas under the microscope) at 400×, and an average percentage of positive-stained cells was determined. The immunostaining of each section was evaluated by two experienced oral and maxillofacial pathologists (Y.-K. Chen and S.-H. Hsue), who independently evaluated the staining scores. When disagreement existed amongst the two observers, a consensus was reached by discussion. Inter-observer agreement was evaluated using kappa statistics (20). The kappa

Figure 1 Radiographic evidence of bony invasion of gingival squamous cell carcinoma (SCC) for a representative surgical resected mandibular specimen.



754

value was calculated to assess inter-observer agreement. A kappa value of < 0.40 was considered as showing poor agreement; one of 0.40–0.59, fair agreement; one of 0.60–0.74, good agreement; and one of 0.75–1.00, excellent agreement.

## Results

Inter-observer agreement amongst the two observers was excellent for the assessment of the staining scores, showing a kappa value of 0.89. Moderate to strong cytoplasmic staining for the RANKL proteins was detected in the cancer cells of the 25 cases of buccal SCC studied (Fig. 2A). In addition, the same staining was also observed in the cytoplasm of cancer cells and osteoclasts in all 15 cases that involved mandibular bone invasion (Figs 3A and 4A). In contrast, strong cystoplasmic staining of RANKL was limited to the basal cell layer in all five control cases of normal buccal mucosa (Fig. 5A).

A similar staining pattern has been noted for RANK protein in all specimens of buccal SCC without bony invasion (Fig. 2B) and gingival SCC with mandibular bony invasion (Figs 3B and 4B). In contrast, there was a complete absence of staining in all the normal mucosal tissues (Fig. 5B). Furthermore, only weak to negative cytoplasmic stained OPG protein was found in any of the specimens of buccal SCCs without bony invasion (Fig. 2C) or gingival SCCs with mandibular bony invasion (Figs 3C and 4C), but was absent in the normal mucosal tissues (Fig. 5C).

The average total scores for RANKL, RANK, and OPG immunostainings of all the samples are summarized in Table 1. The average total scores for both RANKL and RANK immunostainings were significantly higher than the average total score of OPG staining, respectively, for both buccal SCCs without bony invasion and gingival SCCs with mandibular bony invasion (P < 0.0001). On the other hand, the average total scores for RANKL, RANK, and OPG immunostainings of both buccal SCCs without bony invasion and gingival SCCs with mandibular bony invasion were, respectively, significantly higher than the average total score of the normal tissues (P < 0.0001).

## Discussion

Both RANKL [also known as osteoclast differentiation factor (6)] and OPGL are the most potent factors for inducing osteoclastogenesis (6, 7), and RANKL-deficient mice demonstrate an osteopetrotic phenotype (21). There are two types of RANKL (membrane-bound and soluble types), but the functional differences between them are unclear (21). The signaling receptor for RANKL is RANK, which belongs to the TNF family (11). The natural decoy receptor for RANKL is OPG, and osteoclast formation is regulated by the balance between OPG and RANKL (14).

Following a review of the English-language medical literature, to the best of our knowledge, this study is the first *in vivo* analysis of RANKL, RANK, and OPG

expression in human SCC of the oral cavity. In this study, an up-regulation of RANKL/RANK expression has been demonstrated in both the buccal SCC without bony invasion and oral SCC with such invasion, compared with normal oral mucosa. Furthermore, only weakly stained OPG protein was noted in both SCC



**Figure 2** Representative sample of a buccal SCC without bony invasion. Strong cytoplasmic staining of RANKL (A) and RANK (B) proteins was detected in the cancer cells; a weak cytoplasmic staining of OPG was also noted (C) (ABC stain  $\times 100$ ).



Figure 3 Representative sample of a gingival SCC with bony invasion. Strong staining of RANKL (A) and RANK (B) proteins was detected in the cytoplasm of the cancer cells; but there is a negative OPG staining (C) (ABC stain  $\times$ 40).



Figure 4 Representative sample of a gingival SCC with bony invasion. Strong staining of RANKL (A) and RANK (B) proteins was detected in the cytoplasm of the osteoclasts; but there is a weak OPG staining (C) (ABC stain ×200).

with and without osseous destruction, indicating a very low concentration of OPG. This change would allow an increased rate of RANK binding to RANKL. These findings are largely compatible with the two previous *in vitro* studies (15, 16). Taking the *in vivo* and *in vitro* data together, it appears that human oral SCC cells with and without osseous destruction can express RANKL, RANK, and OPG proteins. This finding is consistent with previous reports on other types of human cancer, such as breast cancer (22), prostate cancer (23), and myeloma (24), that also noted the role of the RANKL/RANK signaling pathway in these three cancers.

In this study, we have found that there are no differences between the immunohistochemical expression of RANKL, RANK, and OPG in cases of buccal SCC without bony invasion and oral SCC with such invasion (with the exception of the presence of

RANKL/RANK/OPG in oral carcinoma Chuang et al.



**Figure 5** Representative sample of a normal buccal mucosa. Strong cytoplasmic staining of RANKL (A & inset) was noted only in the basal cell layer, and there is a negative staining of RANK (B) and OPG (C) (ABC stain ×100).

Table 1	The average total	scores $(S)$	for the	immunostainings	of RANKL	RANK	and OPG	of this study
I HOIC I	The average total		ioi the	minunostamingo	or running.	,	, una or o	or time study

		Average total scores (S)	
	RANKL	RANK	OPG
Buccal SCCs without bony invasion Gingival SCCs with bony invasion Normal tissues	$\begin{array}{rrrr} 7.20 \ \pm \ 1.00^{a,b} \\ 7.47 \ \pm \ 0.92^{a,b} \\ 1.40 \ \pm \ 0.55 \end{array}$	$\begin{array}{rrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrr$	$\begin{array}{rrrr} 3.52 \ \pm \ 0.51^{\rm b} \\ 3.73 \ \pm \ 0.46^{\rm b} \\ 0 \ \pm \ 0.0 \end{array}$

RANKL, receptor activator of NF-KB ligand; OPG, osteoprotegerin; SCC, squamous cell carcinoma.

S = P (percentage of stained cells) × I (Intensity of stained cells), as stated in Materials and methods.

 ${}^{a}P < 0.0001$  compared with OPG group;  ${}^{b}P < 0.0001$  compared with normal tissues.

multinucleated osteoclasts for oral SCC with bony invasion). These findings suggest that for those cases of human buccal SCC without bony invasion, they do actually possess the potential to induce osteoclastogenesis through the RANKL/RANK/OPG pathway if triggered under appropriate conditions. It is possible that close approximation of the cancer cells with the jaw bone may be a prerequisite (3, 4). Another commentary (24) has indicated that cell-to-cell contact between cancer cells and host cells may not always lead to RANKL expression; the degree of up-regulation of RANKL/RANK expression may also be variable. This correlates with clinical observations, as not all SCCs of the oral cavity approximating bone show osseous destruction. Unfortunately, our immunochemical investigation is unable to provide a precise measure of the level of such up-regulation. To define the veracity of such an index, further functional assays on knockdown of components of the RANKL/RANK/OPG pathway and animal studies may be of benefit. Such advanced studies could establish the conditions required to activate osteoclastogenesis via the RANKL/RANK/OPG pathway.

This study is, to the best of our knowledge, the first to demonstrate the expression of RANKL, RANK, and OPG in normal human oral mucosa. Strong cytoplasmic staining of RANKL has been noted in the basal cell layer of normal mucosa, and there is negative staining of RANK and OPG. On the other hand, given that an upregulation of cytokines (including TNF) has already been identified in gingival SCC (25), one could hence speculate that during oral carcinogenesis, RANK (a TNF receptor) becomes up-regulated in cancer cells (as noted in this study).

In conclusion, an enhanced immunohistochemical expression of RANKL, RANK, and OPG in the cytoplasm of human SCC compared with that in normal mucosa of the oral cavity has been demonstrated. This suggests the possible value of the RANK/RANK-L/OPG pathway as biomarkers in human oral SCCs.

## References

- Jordan RC, Daley T. Oral squamous cell carcinoma: new insights. J Can Dent Assoc 1997; 63: 517–8.
- Chen YK, Huang HC, Lin LM, Lin CC. Primary oral squamous cell carcinomas: an analysis of 703 cases in southern Taiwan. *Oral Oncol* 1999; 35: 173–9.
- 3. Brown JS, Lowe D, Kalavrezos N, D' Souza J, Magennis P, Woolgar J. Patterns of invasion and routes of tumor entry into the mandible by oral squamous cell carcinoma. *Head Neck* 2002; **24**: 370–83.
- 4. Wong RJ, Keel SB, Glynn RJ, Varvares MA. Histological pattern of mandibular invasion by oral squamous cell carcinoma. *Laryngoscope* 2000; **110**: 65–72.
- 5. Semba I, Matsuuchi H, Miura Y. Histomorphometric analysis of osteoclastic resorption in bone directly invaded by gingiva squamous cell carcinoma. *J Oral Pathol Med* 1996; **25**: 429–35.
- 6. Yasuda H, Shima N, Nakagawa N, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 1998; **95**: 3597–602.
- 7. Lacey DL, Timms E, Tan HL, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998; **93**: 165–76.
- Wong BR, Josien R, Lee SY, et al. TRANCE (tumor necrosis factor (TNF)-related activation induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med* 1997; **186**: 2075–80.
- 9. Hsu H, Lacey DL, Dunstan CR, et al. Tumor necrosis factor receptor family member RANK mediates osteoclast

differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci USA* 1999; **96**: 3540–5.

- Horowitz MC, Xi Y, Wilson K, Kacema MA. Control of osteoclastogenesis and bone resorption by members of the TNF family of receptors and ligands. *Cytokine Growth Factor Rev* 2001; 12: 9–18.
- Simonet WS, Lacey DL, Dunstan CR, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997; 89: 309–19.
- Zhang H, Yano S, Miki T, et al. A novel bisphosphonateminodronate (YM529) specifically inhibits osteolytic bone metastasis produced by human small-cell lung cancer cells in NK-cell depleted SCID mice. *Clin Exp Metastasis* 2003; **20**: 153–9.
- 13. Yano S, Zhang H, Hanibuchi M, et al. Combined therapy with a new bisphosphonate, minodronate (YM529), and chemotherapy for multiple organ metastasis of small cell lung cancer cells in severe combined immunodeficient mice. *Clin Cancer Res* 2003; **9**: 5380–5.
- 14. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *J Bone Miner Res* 2000; **15**: 2–12.
- 15. Nagai M, Kyakumoto S, Sato N. Cancer cells responsible for humoral hypercalcemia express mRNA encoding a secreted form of ODF/TRANCE that induces osteoclast formation. *Biochem Biophys Res Commun* 2000; **269**: 532–6.
- Tada T, Jimi E, Okamoto M, Ozeki S, Okabe K. Oral squamous cell carcinoma cells induce osteoclast differentiation by suppression of osteoprotegerin expression in osteoblasts. *Int J Cancer* 2005; **116**: 253–62.
- 17. Sobin LH, Wittekind C. International Union Against Cancer (UICC), TNM classification of malignant tumors, 6th edn. New York, NY: Wiley-Liss, 2002.
- Hsu SM, Raine L, Fanger H. Use of avidin-biotinperoxidase complex (ABC) in immunoperoxidase techniques. J Histochem Cytochem 1981; 29: 577–80.
- Sarbia M, Loberg C, Wolter M, et al. Expression of bcl-2 and amplification of *c-myc* are frequent in basaloid squamous cell carcinomas of the esophagus. *Am J Pathol* 1999; **155**: 1027–32.
- 20. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; **331**: 159–74.
- 21. Kong YY, Yoshida H, Sarosi I, et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 1999; **397**: 315–23.
- 22. Thomas RJ, Guise TA, Yin JJ, et al. Breast cancer cells interact with osteoblasts to support osteoclast formation. *Endocrinology* 1999; **140**: 4451–8.
- Brown JM, Corey E, Lee ZD, et al. Osteoprotegerin and rank ligand expression in prostate cancer. *Urology* 2001; 57: 611–6.
- 24. Sezer O, Heider U, Zavrski I, Kuhne CA, Hofbauer LC. RANK ligand and osteoprotegerin in myeloma bone disease. *Blood* 2003; **101**: 2094–8.
- Shibahara T, Nomura T, Cui NH, Noma H. A study of osteoclast-related cytokines in mandibular invasion by squamous cell carcinoma. *Int J Oral Maxillofac Surg* 2005; 34: 789–93.

758