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Functional Biomarkers of Oral Cancer

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1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common head and neck cancers, and rank as one of the top ten cancers worldwide. More worrying is that the incidence of oral cancer appears to be increasing in many parts of the world. OSCC is characterized by a high degree of local invasiveness and a high rate of metastasis to the cervical lymph nodes. Survival of patients with OSCC has not improved in the last 40 years, despite recent advances in surgical procedures and the availability of new chemotherapeutic agents. In addition, surgical resection results in significant functional and cosmetic defects; therefore, it is important to develop conservative therapeutics, whereupon identification of markers representing OSCC aggressiveness would be worthwhile to decide the most suitable treatment for each patient from therapeutic options.

Given that the head and neck region is an environment challenged by a large variety of insults, including pathogens, foods, and chemicals, the relationship between cancer cells and inflammatory stroma might be of particular importance for arising malignancies there. The microenvironment plays a critical role in tumor initiation and progression, and may provide attractive therapeutic targets. In fact, it affects not only tumor growth, invasion, and metastasis, but also drug metabolism and accessibility; hence the role of stromal elements has been extensively investigated at a molecular level. Although host-tumor interactions are two-way communications between cancer cells and stroma; however, knowledge about the cancer cell properties specifically evoked in the microenvironment is still limited.

In this review, we provide an overview of the literatures regarding “functional biomarkers” of OSCCs, introduce our recent discovery of the *in vivo*-specific maker, and discuss significance of these factors in diagnostic and clinical implication.

2. Cellular biomarkers in OSCC

Major cellular biomarkers correlated with the clinical outcome of OSCC have been reported, which were refined with a focus on the relationship between prognostic or survival parameters of OSCC patients and their expression levels, mainly using immunohistochemistry (Oliveira & Ribeiro-Silva, 2011). The biomarkers could be classified into five groups based on their biological functions: 1) cell cycle progression and

proliferation; 2) tumor suppression and apoptosis; 3) hypoxia; 4) angiogenesis; and 5) cell adhesion and matrix degradation.

2.1 Cell cycle progression and proliferation biomarkers

Several biomarkers belonging to this group have been identified: a family of epidermal growth factor receptors (EGFRs); cyclin D1; cyclin B1; Ki-67; proliferating cell nuclear antigen (PCNA); and Akt1. The EGFR family includes four members: HER-1 (EGFR, ErbB1), HER-2 (neu/ErbB2), HER-3 (ErbB3), and HER-4 (ErbB4). EGFR activation, as its name indicates, can augment the malignant potential of epithelial cells (LaCasse et al., 2008), and the overexpression correlates with poor prognosis in OSCC patients (Agra et al., 2008; Laimer et al., 2007; Silva et al., 2008, 2009). Cyclin proteins are key regulators of cell cycle progression. Cyclin D1 amplification is frequently detected as molecular alterations in OSCC and other head and neck cancers (Yu et al., 2005). There is a significant association between the combined expression of EGFR, cyclin D1, and p53 and an unfavorable overall survival in OSCC patients (Shiraki et al., 2005). Cyclin B1 is useful in predicting occult cervical lymph-node metastasis in OSCC (Harada et al., 2006). Ki-67 and PCNA are well-established cell proliferation markers, and Ki-67 expression increases sharply in early OSCC, but significantly decreases during disease progression (Derka et al., 2006), while that of PCNA was not significantly associated with the survival (Kim et al., 2007; Lim et al., 2005; Myoung et al., 2006). Akt plays a pivotal role in cell survival and proliferation. Overexpression of Akt is a significant indicator for predicting poor prognosis in OSCC patients (Lim et al., 2005).

2.2 Tumor suppression and apoptosis biomarkers

Five subsets of biomarkers were identified in this group: p53/p63, p21/p27, Bcl-2 family members, the retinoblastoma (Rb) protein, and Survivin. The tumor suppressor p53 is one of the most studied biomarkers in OSCC, as well as in other malignancies. The high expression of p53 is especially detected at advanced stages of carcinogenesis, and is also associated with a poor prognosis (Oliveira et al., 2007a, 2007b, 2008), while the clinical significance of that of p63, a p53 homologue, remains controversial (Oliveira et al., 2007a, 2007b; Lo Muzio et al., 2005a, 2007). Mutation of p53 occurs in 50% of OSCC cases (Ogden et al., 1992, 1996), which might be pertinent to consequent overexpression of this molecule. p21^{waf1/cip1} and p27^{Kip1} play an important function in regulating cell cycle progression through the inhibitory action on cyclin-dependent kinases. There are controversial findings concerning the clinical outcome of p21^{waf1/cip1}-positive OSCCs (Fillies et al., 2007; Nemes et al., 2005), whereas no significant association between p27^{Kip1} expression and OSCC prognosis has been identified (Fillies et al., 2007). Bcl-2 family members include both anti- and pro-apoptotic proteins, and thereby regulate apoptosis either positively or negatively through their balance (Camisasca et al., 2009). The survival rate of patients with Bcl-2-negative and Bax-positive OSCC tumors was significantly higher than those with other expression profiles of these molecules (Kato et al., 2008; Zhang et al., 2009). The Rb pathway plays a crucial role in regulating cell cycle progression. OSCCs lacking Rb indeed display aggressiveness with the poor prognosis (Soni et al., 2005). Survivin is an inhibitor of apoptosis and overexpressed in most of OSCCs, indicating a potential biomarker of aggressiveness and invasiveness (Lippert et al., 2007; Lo Muzio et al., 2005b).

2.3 Hypoxia biomarkers

Four hypoxia-related biomarkers have been reported as putative prognostic parameters: hypoxia inducible factor 1 α (HIF-1 α); carbonic anhydrase IX (CA IX); glucose transporter 1 (GLUT-1); and erythropoietin receptor (EPOR). In hypoxic environments, stabilized HIF-1 α induces the transactivation of more than 70 genes, including vascular endothelial growth factor (VEGF), involved in hypoxia adaptation and/or reversion (Fillies et al., 2005; Liu et al., 2008). The diffuse overexpression of HIF-1 α had been associated with a good prognosis in OSCC patients (Fillies et al., 2005), whereas recently the opposite evidences have been reported (Lin et al., 2008; Liu et al., 2008). The latter finding might be supported by the ability of HIF-1 α in inducing VEGF and promoting invasive phenotypes. Indeed, HIF family members are implicated in epithelial mesenchymal transition (EMT) of cancer cells (Yang MH., 2008). CA IX, a member of HIF-1-dependent CA family, is a transmembrane glycoprotein involved in pH homeostasis (Sakata et al., 2008). Overexpression of CA IX has been shown to significantly associate with recurrence and worse survival in OSCC patients (Choi et al., 2008). GLUT is also regulated via the HIF-1 pathway, mediates cellular glucose uptake, and namely represents as an endogenous marker of hypoxia (Kunkel et al., 2007). There is evidence that GLUT-1 is associated with aggressiveness in OSCC (Jonathan et al., 2006). Erythropoietin (EPO) is a glycoprotein hormone and synthesized in the kidneys in response to hypoxemia, and OSCC has been known to express its cognate receptor EPOR (Arcasoy et al., 2005). High expression of EPOR can be associated with a significantly worse prognosis in patients with oral tongue squamous cell carcinoma (Roh et al., 2009).

2.4 Angiogenesis biomarkers

Four angiogenic biomarkers were identified as possible prognostic parameters: VEGF; endoglin (CD105); CD34; and Eph receptor tyrosine kinases. VEGF functions mainly as an angiogenic cytokine that promotes proliferation, differentiation, and migration of vascular endothelial cells. Therefore, it should be ideally discussed in the section of humoral factors, but we would like to state here due to the importance of its intracellular transcriptional regulation by HIF-1. The patients with VEGF-positive tumors have significantly poor survival (Chien et al., 2006; Shao et al., 2008). Despite the established role of VEGF for angiogenesis, a recent study showed that an anti-VEGF antibody failed to interrupt the connection between OSCC and endothelial cells (Yamada et al., 2011); hence VEGF functions in tumor progression, other than angiogenesis, have yet to be determined. CD105, a regulatory component of the TGF- β receptor complex, can modulate angiogenesis. High expression of CD105 in primary OSCC may identify patients at risk of the recurrence with worse prognosis (Chuang et al., 2006). In addition, the existence of penetrating vessels within tumor nests, endothelial cells of which can be visualized by CD34 staining, was significantly associated with risk of cervical lymph node metastasis (Kademani et al., 2009). The Eph receptors and their membrane-anchored ephrin ligands can stimulate invasive behavior in a tumor through a cell-cell communication system capable of bi-directional signaling, thereby promoting a more aggressive and metastatic phenotype (Campbell et al., 2008; Wimmer-Kleikamp et al., 2005). High expression of EphA2 was associated with a poor survival in tongue SCC patients (Shao et al., 2008).

Category (section no.) Pathways (section no.)	Biomarker	Status	Cellular/clinical characteristics
Cellular Biomarker (2)			
<i>Cell cycle promotion and proliferation (2.1)</i>			
	EGFR	overexpression	poor prognosis
	Cyclin D1	amplification	unfavorable overall survival
	Cyclin B1	overexpression in cytoplasm	cervical lymph node metastasis
	Ki-67	increase (early), decrease (late)	poor overall survival
	PCNA	positive	no significant association on survival
	Akt	overexpression	poor prognosis
<i>Tumor suppression and apoptosis (2.2)</i>			
	p53	overexpression, mutation	poor prognosis
	p63	positive	controversial
	p21	overexpression	controversial
	p27	overexpression	no significant association on survival
	Bcl-2	negative	higher survival
	Bax	positive	higher survival
	Rb	loss	malignant conversion, poor prognosis
	survivin	overexpression	aggressive and invasive
<i>Hypoxia (2.3)</i>			
	HIF-1 α	diffuse overexpression	good prognosis or controversial
	CA IX	overexpression	recurrence with worse survival
	GLUT-1	high expression	metastasis, worse overall survival
	EPOR	high expression	worse prognosis
<i>Angiogenesis (2.4)</i>			
	VEGF	positive	poor prognosis
	CD105	high expression	recurrence with worse prognosis
	CD34	positive within tumor nests	cervical lymph node metastasis
	Eph A2	high expression	poor survival
<i>Cell adhesion and matrix degradation (2.5)</i>			
	MMP-7,-9, -13, -14	positive	poor prognosis
	CD44	irregular cytoplasmic staining	poor overall survival
	E-cadherin	downregulation	EMT, worse prognosis
	N-cadherin	upregulation	EMT
	β - and γ -catenin	positive	poor prognosis
	versican	overexpression in stroma	unfavorable outcome
Humoral Biomarker (3)			
<i>Parathyroid hormone-related protein (3.1)</i>			
	PTHrP	high expression	malignant conversion
<i>Endothelins and their receptors (3.2)</i>			
	Endothelins (ETs)	overexpression	tumor growth and progression
	ET _A R, ET _B R	overexpression	tumor growth and progression
<i>Inflammatory Cytokines and Chemokines (3.3)</i>			
	Interleukin (IL)-6	high expression	tolerance to immune system
	IL-8	high expression	controversial
	TNF- α	high expression	controversial
	CXCL13	high expression	tumor development and progression
In vivo specific Biomarker (4)			
<i>Receptor activator of NF-κB ligand</i>			
	RANKL	high expression <i>in vivo</i>	tumor development and progression

Table 1. Molecular biomarkers and its correlation with cellular or clinical characteristics.

2.5 Cell adhesion and matrix degradation biomarkers

Five classes of matrix degradation- and cell adhesion-related molecules were identified as putative prognostic biomarkers associated with OSCC: matrix metalloproteinases (MMPs), CD44, cadherins, catenins, and versican. The MMPs are a family of proteases, highly expressed by invasive tumor cells and the adjacent stroma, and essential for ECM degradation (de Vicente et al., 2005). Of over 20 known members of MMPs, expression of MMP-7, -9, -13, and -14 (MT1-MMP) were significantly associated with poor prognosis of OSCC patients (De Vicente et al., 2005, 2007; Luukkaa et al., 2006). The CD44 family is widely expressed transmembrane glycoproteins that bind to hyaluronic acid, growth factors, and ECM proteins, regulating cell migration and adhesion (Georgolios et al., 2006). In contrast to strong membranous staining in normal squamous cell epithelium, the irregular cytoplasmic staining of CD44 in OSCC has been shown to correlate with poor disease-free and overall survivals (Kosunen et al., 2007). Cadherins are a family of transmembrane glycoproteins involved in cell-cell adhesion (Munoz-Guerra et al., 2005). In most epithelial cells, the intracellular domain of E-cadherin binds to catenins, forming the cadherin-catenin complex involved in the intracellular transduction of cell-to-cell contact signals. The reduced expression of E-cadherin, frequently concomitant with N-cadherin upregulation, leads to loss of cell-cell adhesion and acquisition of the mesenchymal phenotype (i.e. EMT), which plays an important role in tumor invasion and dissemination (Lyons et al., 2007). Accordingly, a decrease in the expression of β - and γ -catenins can also predict poor prognosis of OSCC (Ueda et al., 2006). Versican is a major proteoglycan of the ECMs, and its overexpression was observed in diverse tumors. This molecule plays an essential role in tumor growth by repressing cell adhesion, stimulating cell proliferation and migration, and regulating angiogenesis (Rahmani et al., 2006). High stromal versican expression in OSCC specimens is an independent predictor for an unfavorable prognosis (Pukkila et al., 2007).

3. Humoral biomarkers in OSCC

Given that the significance of the microenvironment for OSCC initiation and/or promotion has been shown, as mentioned above, humoral factors that correlate with clinical features would be of particular importance. VEGF may serve as a prototype molecule bridging between cancer cells and stromal component, namely endothelial cells as described (see 2.4). In this section, humoral biomarkers are enumerated irrespective of their source and their mode of action, para- or autocrine.

3.1 Parathyroid hormone-related protein (PTHrP)

PTHrP was originally identified as a major factor responsible for humoral hypercalcemia in malignancies (Burtis et al., 1990), and acts as a stimulator of osteoclastic bone resorption (Liao & McCauley, 2006). Therefore, plasma PTHrP level can be a predictor of existence of bone metastatic lesions in a wide range of tumors. PTHrP produced by cancer cells promotes malignant conversion (increased cell proliferation, survival, adhesion, migration, and invasion) of breast, colon, and prostate cancers (Shen et al., 2004), as well as OSCC (Nomura et al., 2007). Because OSCC expresses the PTH/PTHrP receptor PTH1R, its mechanism of action is in a paracrine or autocrine manner (Yamada et al., 2008). It is noteworthy that the expression level of PTHrP is regulated by downstream signaling of EGFR, another class of biomarker for malignant potential of OSCC (see 2.1). In addition, a

recently developed, rapid screening system identifies PTHrP as one of the validated predictors for OSCC (Ziober et al., 2006).

3.2 Endothelins and their receptors as biomarkers in OSCC

Hoffmann et al. recently reported novel functional biomarkers in OSCC, endothelin (ET) and its receptor, which are overexpressed in OSCC (Hoffmann et al., 2010). ETs comprise a family of three small peptides: ET-1, ET-2, and ET-3 (Yanagisawa et al, 1988; Levin, 1995). ET-1 is expressed primarily in endothelial cells, and ET-2 is in the kidneys and the intestine, whereas ET-3 is found mainly in the brain (Levin, 1995). ETs exert their effects by binding to cell-surface receptors, namely ET-A (ET_AR) and ET-B (ET_BR). Both receptors belong to the G-protein-coupled receptor super-family (Levin, 1995; Kusserow et al., 2004; Motte et al, 2006; Bhalla et al., 2009). ET_AR binds ET-1 with 10-times greater affinity than ET-3, whereas ET_BR binds all three ETs with similar affinity. In general, most ET-1 functions are therefore mediated by interaction with ET_AR (Guise et al., 2003).

ET-1, ET_AR, and ET_BR are overexpressed in OSCC, in which ET-1 acts as a survival factor to induce proliferation via ET_AR and ET_BR (Awano et al., 2006). Schmidt et al. demonstrated a significant elevation in the levels of ET-1 in HSC-3 cells (Schmidt et al, 2007), a lineage obtained from human OSCC. ET_AR activation by ET-1 largely contributes to tumor growth and progression through induction of cell proliferation, survival, angiogenesis, and metastatic spread, thus indicating that ET_AR antagonism might improve cancer treatment (Rosano et al., 2006; Nelson et al, 2003). ET-1 can also modulate tumor angiogenesis through induction of VEGF, which is accounted for by an increase in HIF-1 α level by ET_AR activation (Bagnato et al, 2002). Besides their anti-angiogenic effect, ET receptor antagonists can also prevent the production of MMPs from macrophages (Grimshaw, 2007). Taken together, it is tempting to infer that blocking ET receptors, especially ET_AR, might be a useful alternative as an adjuvant treatment of OSCC. Nevertheless, whether ET antagonists provide significant clinical benefit for patients with OSCC is a vital and controversial issue. In several clinical trials of metastatic castration-resistant prostate cancer patients, atrasentan, a competitive inhibitor of ET-1, did not improve the primary or secondary endpoint (Carducci, 2007; Nelson, 2008). Similar results have been obtained in other studies using a different ET antagonist zibotentan. Therefore, results of adequate, long-term clinical trials for OSCC are awaited.

3.3 Inflammatory cytokines and chemokines

Given the specific property of the oral cavity that is always challenged by a large variety of insults, including pathogens, foods, and chemicals, chronic inflammation and its molecular components such as cytokines or chemokines would be particularly important for the development and progression of OSCC. In fact, several cytokines and chemokines are identified as biomarker of OSCC.

Interleukin (IL)-6 and IL-8 have been implicated as potential biomarkers for OSCC. (St John et al., 2004). When these cytokines are expressed together with VEGF, OSCC has been shown to acquire resistance in a manner dependent on immune effectors (Teruel et al., 2008). In addition, the concentration of saliva IL-6 in OSCC patients are significantly higher than that of control group, whereas results regarding IL-8 and tumor necrosis factor- α are controversial (SahebJamee et al., 2008).

Chemokines are also implicated in tumor progression and metastasis of OSCC (Krieg & Boyman, 2008). More recently, gene expression profiling studies implicated chemokine ligand-13 (CXCL13) in OSCC tumor development and progression (Ziober et al., 2006). CXCL13 (BCA-1) that binds monogamously to the CXCR5 receptor was originally discovered to facilitate B-cell chemotaxis (Legler et al., 1998). CXCL13 can upregulate receptor activator of NF- κ B ligand (RANKL), a member of the tumor necrosis factor family critical for osteoclastogenesis (Hsu et al., 1999), through activation of c-Jun N terminus kinase and nuclear factor of activated T cells (NFAT)-4, implicating CXCL13 as a potential biomarker to predict OSCC bone invasion or osteolysis (Yuvaraj et al., 2009).

4. *In vivo*-specific biomarkers — Lessons from RANKL

Survival of patients with OSCC has not improved over the past few decades, despite recent advances in the treatments. One of the fundamental factors explaining the poor outcome is that a great proportion of oral cancers are diagnosed at advanced stages. In addition, surgical resection results in significant functional and cosmetic defects. Therefore, it is important to develop biomarkers for early diagnosis, as well as for the prediction of disease progression and/or aggressiveness to decide the most suitable treatment for each patient from therapeutic options. These facts have prompted molecular exploration for novel markers, and indeed lead to the discovery of manifold biomarkers, as described in the previous sections.

Meanwhile, if a marker were functional in tumorigenesis, aggressiveness, or progression, it can be a plausible therapeutic target. As far as now, most markers have been identified because of their high expression in tumor tissues, evaluated by immunohistochemistry, and the significance are evaluated by comparison with clinical course of the patients, followed by functional analysis *in vitro*. However, given the specific feature of the oral cavity that is an environment challenged by a large variety of insults, including pathogens, foods, and chemicals, the molecules exert their functions *in vivo* would be more preferable.

We have recently identified the osteoclastic cytokine RANKL as a marker for invasive and aggressive OSCC with its molecular function as an EMT and angiogenesis inducer (Yamada et al., 2011). Of particular interest is that both RANKL expression and function solely depend on the microenvironment; neither its high expression nor function, as the EMT inducer could not be reproduced *in vitro*. In other word, it is not necessarily enough to explore molecular functions only using *in vitro* settings. In the latter part of this review, we highlight the story of how RANKL is characterized as an *in vivo*-specific biomarker with the experimental procedures used, so that such attractive and valuable candidates for biomarker would not be abandoned because of the absence of *in vitro*-experimental evidence, as hypothesized

4.1 High expression of RANKL in *in vivo* OSCC but not in cell lines

We initially probed bone invasion-related factors in OSCC, and indeed reported that PTHrP is expressed in OSCC and enhances the malignant potential of OSCC (Yamada et al., 2008). We therefore hypothesized that PTHrP induces the expression of RANKL in a manner analogous to osteoblasts (Leibbrandt & Penninger, 2008; Mundy, 2002; Roodman, 2004), and examined the expression of RANKL mRNA and protein by quantitative RT-PCR and immunohistochemistry, respectively, in 20 human OSCC samples, including those in the

tongue and the gingiva. In all cases, high expression (from 6 to 123-fold) was observed compared to human control gingival fibroblast (Figure 1A), and the expression level of RANKL was positively correlated with the histological grading of differentiation and invasive histological architecture based on Yamamoto-Kohama classification (Yamamoto et al., 1983). In addition, abundant RANKL protein was observed in atypical cancer cells diffusely invaded into surrounding tissues. Nevertheless, none of the tested OSCC cell lines displayed such abundant RANKL expression as that observed *in vivo*. In particular, cell lines established from poorly differentiated SCC with aggressive invasiveness failed to do so. These results raise the possibility that the RANKL expression level in cell lines is repressed under culture conditions, and RANKL is a microenvironment-induced cytokine *in vivo*, of which expression is implicated in progression and biological malignancies of OSCC.

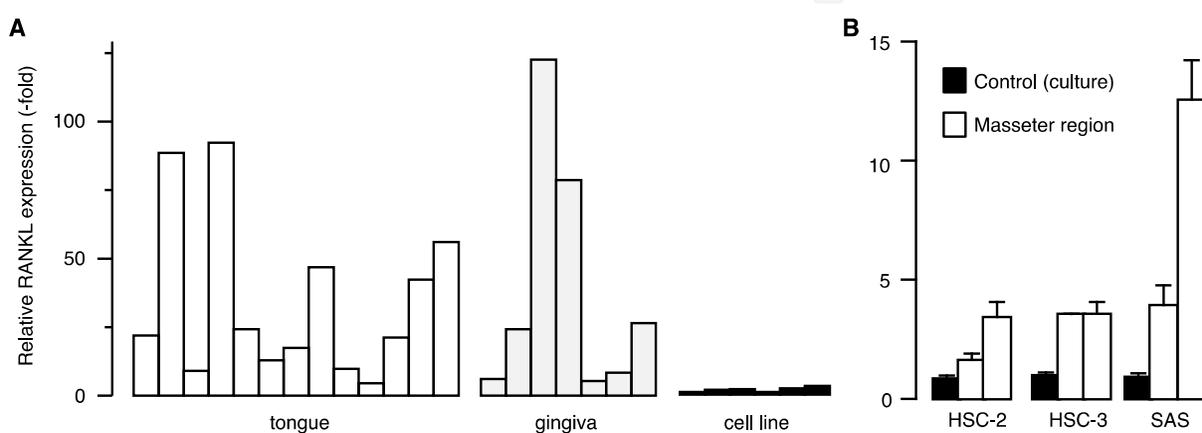


Fig. 1. High RANKL expression *in vivo*. (A) mRNA levels in human OSCC specimens of tongue and gingival cancers and OSCC cell lines were evaluated by quantitative PCR. (B) Cells of OSCC cell lines were inoculated into nude mice and allowed to form tumor. The mRNA level in each sample was determined by quantitative PCR.

4.2 Environment-dependent expression of RANKL

To test the aforementioned hypothesis, several OSCC cell lines were inoculated into the masseter muscles of mice, one of the most established sites for an oral cancer orthotopic model (Cui et al., 2005; Nomura et al., 2007; Suda et al., 1997). As expected, RANKL expression was dramatically augmented at both mRNA and protein levels compared to those in cultured cells (Figure 1B). Moreover, the higher RANKL expression levels are observed in poorly differentiated, invasive SCC, in accordance with our clinicopathological findings.

To assess the contribution of the oral environment to RANKL expression and subsequent tumor formation, HSC-3 cells, a OSCC cell line that formed tumors most efficiently in the orthotopic model, were also injected into the muscle of hindlimbs. This region was selected in analogy to the orthotopic site (intramuscular), as well as by its anatomical location far from the oral cavity. Tumors formed in the hindlimbs were significantly smaller than those in the masseter region (Figure 2A and B). In parallel with their tumor weight, RANKL could be detected in the masseter region tumors, but not in the hindlimb ones (Figure 2C). Thus, RANKL expression requires the orthotopic environment and correlates with tumor formation ability. Moreover, masseter region tumors, but not hindlimb ones, displayed the histological pattern of poorly differentiated squamous cell carcinoma (Figure 2D).

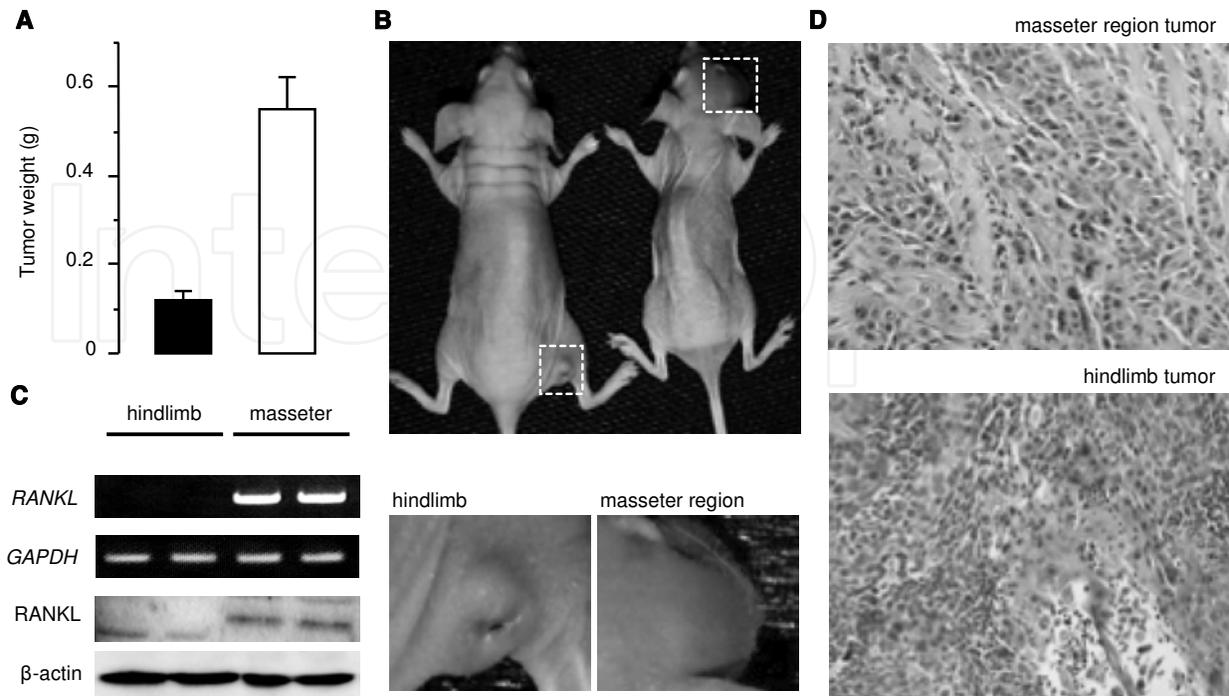


Fig. 2. Environment-dependent expression of RANKL. The OSCC cell line HSC-3 cells were injected into the masseter or hindlimb region of the mice. After 28 days, the formed tumors were weighted (A) and photographed (B). Expression levels of RANKL mRNA and protein in the tumors were analyzed by quantitative RT-PCR and immunoblotting, respectively (C). Histology of the tumors are also shown (D).

4.3 RANKL expression accelerates tumor malignancy

To further verify the role for RANKL in OSCC tumor formation, we established HSC-3 cell lines that stably express RANKL, and injected them into the mouse hindlimbs. RANKL-expressing cells achieved efficient tumor formation in the hindlimbs, whereas control cells failed to form sizable tumors, similar to parental cells as described above (Figure 3A-C). These results together demonstrate that RANKL expression, which ordinarily depends on the oral environment, possesses the potential of inducing OSCC formation. By HE staining, it was revealed that RANKL-expressing, hindlimb-injected tumors exhibited more poorly differentiated and invasive characters than control tumors, consistent with the results observed in human specimens.

4.4 RANKL induces epithelial-mesenchymal transition (EMT)

Immunohistochemical analysis also revealed that E-cadherin was disappeared from the cell-to-cell contact sites in tumors formed by RANKL-expressing cells, whereas control tumors displayed typical E-cadherin pattern (Figure 3D). Moreover, in response to E-cadherin disappearance from the plasma membrane, intense staining for N-cadherin was observed in the tumor cell cytoplasm in RANKL-expressing tumors. These findings raise the possibility that RANKL promotes loss of epithelial character, i.e. evokes EMT, a fundamental process during tumor development and progression (Yang J & Weinberg, 2008).

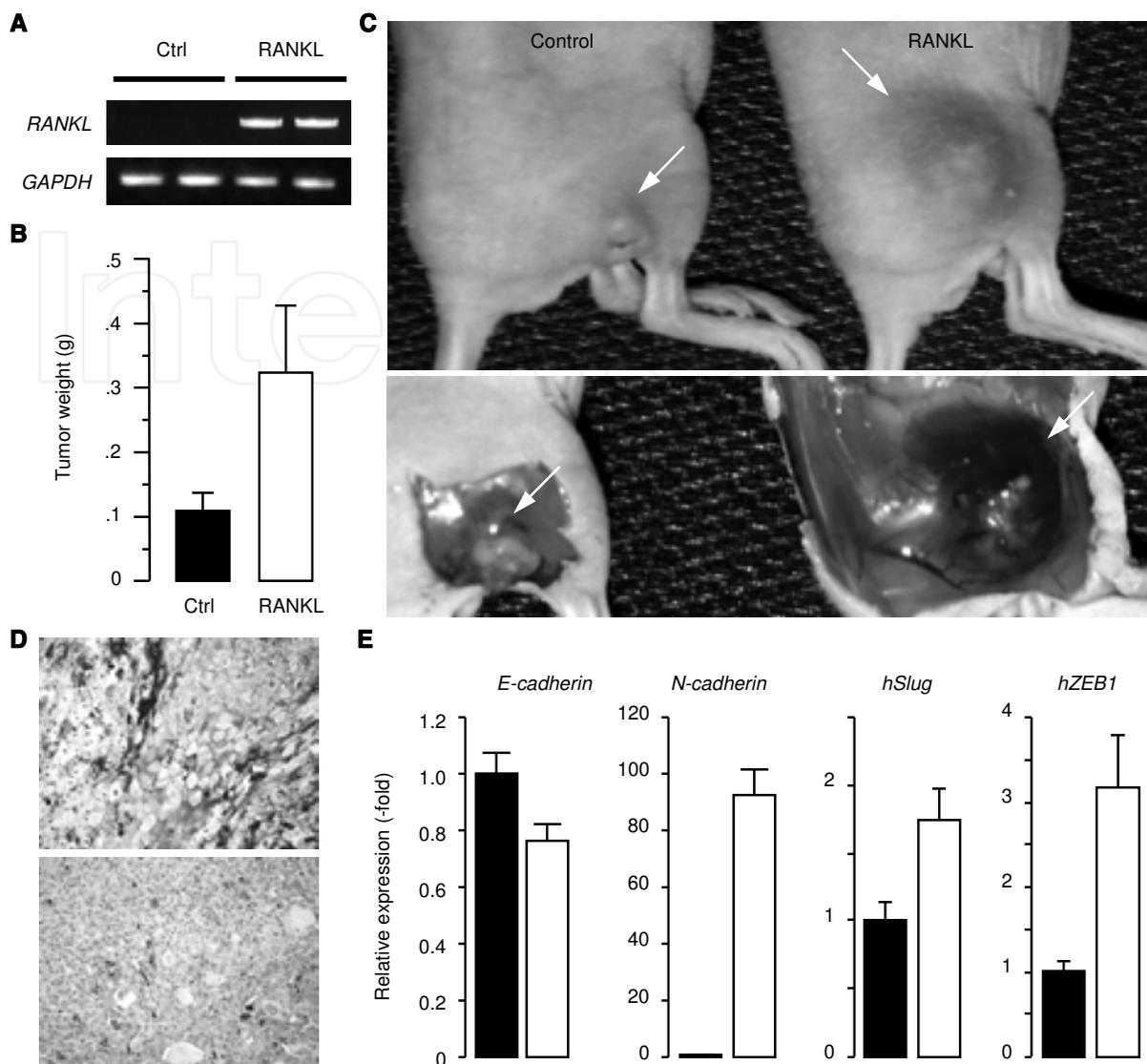


Fig. 3. OSCC tumor formation in hindlimb is facilitated by RANKL. Control (Ctrl) and RANKL-expressing cells (RANKL) were injected into hindlimb muscles and, after 28 days, the tumors were weighed (B), and photographed (C). RANKL mRNA in the tumors was examined by RT-PCR (A). The sections from the control (upper panel) and RANKL-expressing (lower) tumors were subjected to immunohistochemistry using an antibody against E-cadherin (D). mRNA levels of E-cadherin, N-cadherin, Slug, and ZEB1 were also analyzed by quantitative PCR (E).

To test whether RANKL-expressing tumors in fact underwent EMT, we evaluated the expression levels of E-cadherin, N-cadherin, and several transcription factors implicated in EMT (Gotzmann et al., 2004). Indeed, expression of E-cadherin was significantly decreased in RANKL-expressing tumor cells. Accordingly, N-cadherin expression was dramatically upregulated in tumor tissues expressing RANKL. Therefore, these results confirmed that cadherin switching from E-cadherin to N-cadherin occurs in tumors expressing RANKL. Of transcription factors implicated in inducing EMT including Slug, Snail, Twist, and ZEB1, Slug and ZEB1 were upregulated in RANKL-expressing tumors (Figure 3E). However, we could note no differences in their expression *in vitro*. Moreover, notwithstanding the dramatic increment in tumor formation of RANKL-expressing cells in the hindlimbs, there

are no significant differences in *in vitro* proliferation, motility, and invasiveness between RANKL-expressing and control cell lines. Thus, RANKL functions, in addition to its expression, are also in a manner dependent on *in vivo*.

4.5 Significance of RANKL as an *in vivo*-specific biomarker

Beyond its cell autonomous function, RANKL possesses ability to promote tumor angiogenesis, to our surprise, in a manner independent of VEGF. RANKL-expressing tumors were grossly rich in blood vessels, and immunohistochemical analysis using an antibody against CD31, a well-established marker for endothelial cells, revealed that RANKL-expressing tumors harbor significantly more abundant tumor microvessels than control tumors. Interestingly, this angiogenic potency was hampered in the presence of osteoprotegerin (OPG), a RANKL decoy receptor that inhibits RANK-RANKL signaling, whereas neutralization of VEGF using an anti-VEGF antibody failed to do so. These results together demonstrate that RANKL promotes tumor angiogenesis in a manner dependent on its cognate receptor RANK, as reported previously (Kim et al., 2002), but independent of VEGF.

One of the most serious clinical concerns accompanying OSCC is a high potential for local invasion, frequently targeting the adjacent bone. To conquer this, radical and surgical procedures have been enforced; however, the patients suffering from the deprivation of fundamental functions, including mastication and vocalization. Our findings may resolve this longstanding issue in OSCC; the recognition of RANKL and its relevant signaling as potential targets for conservative therapy will enable us to hamper the tumorigenesis and invasion by cutting the connection between OSCC and the tumor microenvironment.

In addition to the conventional molecular targeted therapy (i.e. small compounds and humanized antibodies), RANKL may constitute a better candidate for cancer immunotherapy. Several tumor antigens such as cancer-testis antigens provide specific targets for cancer cells due to their restricted expression patterns (Maio et al., 2003; Nicholaou et al., 2006; Suri, 2006). However, in the case that these molecules are not essential for cancer cell survival, the cells can escape the challenge of the immune system by reducing the expression of the antigens. Since the expression of RANKL in response to the microenvironment is critical for OSCC progression, we strongly propose RANKL-RANK signaling as being central to the conservative, multimodal treatment for this disease.

5. Conclusion

In summary, we have overviewed biomarkers with a particular focus on ones, functions of which are relevant to OSCC development, progression and its malignant potential. As we disclose, *in vivo* "functional biomarkers" such as RANKL would be of particular importance for both diagnosis and therapy of this disease.

The mechanism underlying the microenvironment-specific RANKL expression remains to be addressed. Given that the head and neck regions including the oral cavity are always challenged by every pathogen, the involvement of inflammatory responses might be indispensable for OSCC tumor initiation and progression (Allen et al., 2007; Choi & Myers, 2008; Ferris & Grandis, 2007; Lin et al., 2002; Pries et al., 2006). In addition, this region is also abundant in a range of growth factors that contribute to malignant conversion of OSCC through activating diverse cancer-related signaling pathways (Yamada et al., 2008; Nagano

& Saya, 2004; Ponta et al., 2003; Todd & Wong, 1999). We thus explored RANKL-inducing agents from a wide range of growth factors as well as inflammatory cytokines; however, failed to identify it so far. CXCL13 can certainly upregulate RANKL (Yuvaraj et al., 2009), albeit less efficiently compared to the upregulation observed *in vivo* (unpublished result). In the future, we believe that through our observations and the unveiling of remaining associated issues, the establishment of more rational, potent anti-cancer therapy with consideration of the communication between cancer cells and their respective microenvironment will eventually be accomplished.

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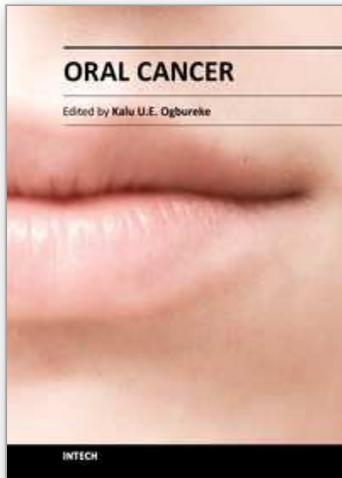
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Oral cancer is a significant public health challenge globally. Although the oral cavity is easily accessible, early diagnosis remains slow compared to the enhanced detection of cancers of the breast, colon, prostate, and melanoma. As a result, the mortality rate from oral cancer for the past four decades has remained high at over 50% in spite of advances in treatment modalities. This contrasts with considerable decrease in mortality rates for cancers of the breast, colon, prostate, and melanoma during the same period. This book attempts to provide a reference-friendly update on the etiologic/risk factors, current clinical diagnostic tools, management philosophies, molecular biomarkers, and progression indicators of oral cancer.

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