Interleukin-1 receptor antagonist (IL1RN) is associated with suppression of early carcinogenic events in human oral malignancies

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Abstract. Inflammatory abnormalities have been implicated in the pathogenesis of various human diseases, including cancer. Interleukin-1 receptor antagonist (IL1RN) is a potent anti-inflammatory molecule that modulates the biological activity of the proinflammatory cytokine, interleukin-1. The aim of this study was to examine the expression of IL1RN in oral squamous cell carcinomas (OSCCs), and to determine its clinical significance. Expression levels of *IL1RN* in matched normal and tumor specimens from 39 OSCCs were evaluated using real-time quantitative polymerase chain reaction methods, and immunohistochemical analysis. Protein expression of IL1RN was also examined in 18 oral premalignant lesions (OPLs). Expression of IL1RN mRNA was significantly downregulated in OSCCs compared with normal tissues. Decreased expression of IL1RN protein was also observed in OPLs and OSCCs. The IL1RN expression level was lower in the OPL cases with severe dysplasia compared to those with mild/moderate dysplasia. Significantly downregulated IL1RN expression was observed in all OSCC lesion sites examined when compared with the matched normal tissues. However, the decreased level of IL1RN expression did not correspond with tumor progression. Noteworthy, IL1RN expression was higher in the advanced OSCC cases (T3/T4) compared to early cases (T1/T2). Among OSCC samples, relatively higher IL1RN expression was associated with active tumor development in the OSCCs occurring in the buccal mucosa, oral floor, fauces and gingiva, but not the tongue. These data suggest that IL1RN may exhibit opposing characteristics in oral malignancies depending on the stage of cancer development, suppressing early carcinogenic events, yet promoting tumor development in some lesion sites. Thus, IL1RN could represent a reliable biomarker for the early diagnosis of OSCCs. Furthermore, IL1RN may possess unknown and complex functions in the developed OSCC.

Introduction

Despite therapeutic and diagnostic advances, oral squamous cell carcinoma (OSCC) patients are often diagnosed at advanced stages, and therefore mortality rates remain high (1). OSCC carcinogenesis appears to evolve through a multistep process involving biomolecular changes, which results in the development of premalignant lesions and, subsequently, an invasive cancer. The identification of the molecular alterations associated with these events could yield insight into the mechanisms underlying the initiation and progression of this neoplasia, and provide new tools for the diagnosis, treatment, and prevention of OSCCs. To address this issue, we recently developed a strategy, using proteomics technologies to search for significant molecular biomarkers for oral carcinogenesis (2). Among the proteins identified was the interleukin-1 receptor antagonist (IL1RN), whose expression was found to be significantly downregulated in OSCC-derived cell lines compared to normal human oral keratinocytes.

It is generally accepted that inflammation is a strong risk factor for tumor development (3,4). In oral cancer, chronic inflammation induced by prolonged exposure to alcohol, tobacco and pathogenic agents, has been considered to represent a potential common denominator in the development of tumors (5,6). Interleukin-1 (IL-1) is a major proinflammatory cytokine, responsible for various acute and chronic inflammatory conditions. Evidence suggests that IL-1 may contribute to the promotion of tumor growth, angiogenesis, and metastasis in various human malignancies (7-9). IL1RN was initially characterized as a naturally occurring antagonist for IL-1. It shares 70% sequence homology with IL-1, and has the ability to bind

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Abbreviations: IL1RN, interleukin-1 receptor antagonist; OSCC, oral squamous cell carcinoma; OPL, oral premalignant lesion

Key words: interleukin-1 receptor antagonist, oral squamous cell carcinoma, oral premalignant lesion

to the membrane-anchored IL-1 receptor without initiating the IL-1 intracellular signaling cascade (10-12). Maintenance of an equilibrium between the IL-1 and IL1RN levels in local tissues influences the relative inflammatory effects of IL-1, and as reviewed by Arend in 2002, alteration of the balance predisposes cells to the development of a variety of diseases, including cancer (13).

Alterations in IL1RN expression could have an important role in oral carcinogenesis, however, little is known about the significance of IL1RN in OSCC. Therefore, the aim of the current study was to investigate the expression levels of IL1RN in a series of human primary OSCCs and oral premalignant lesions (OPLs), in order to establish a link between IL1RN expression and oral malignancies.

Materials and methods

Tissue specimens. Thirty-nine pairs of primary OSCC samples and corresponding normal oral epithelium tissue samples were obtained at the time of surgery performed at Chiba University Hospital between 2005 and 2009. The clinicopathological characteristics of the OSCC cases in this series are summarized in Table I. In addition, the 18 cases of advanced OPLs pathologically diagnosed as leukoplakia with epithelial dysplasia, i.e., mild (n=1), moderate (n=10), and severe (n=7), in a high-risk oral site such as the ventral-lateral tongue or gingiva, were obtained in the same manner. The resected tissues were divided into two parts: one part was frozen immediately after removal of the surrounding normal tissue, and was stored at -80°C until extraction of its RNA was performed, and the second part was fixed in 10% buffered formaldehyde solution in preparation for patho-logical diagnosis and immunohistochemical (IHC) staining. The histopathological diagnosis of each tumor specimen was performed according to the International Histological Classification of Tumors, by the Department of Pathology, Chiba University Hospital. Clinicopathologic staging was determined by the TNM classification system of the International Union against Cancer.

Ethics statement. All patients provided written informed consent, in accordance with the protocol that was reviewed and approved by the Ethical Committee of Graduate School of Medicine, Chiba University (approval number, 236). The study was performed in accordance with the ethical standards of the Declaration of Helsinki.

Preparation of RNA. Total RNA was extracted using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Each extracted RNA or protein sample was stored separately at -80°C until use.

mRNA expression analysis. Following RNA extraction and conversion of the extracted RNA to cDNA by reverse tran-scription, the mRNA expression levels of *IL1RN* were examined by real-time quantitative polymerase chain reaction methods (qRT-PCR) in OSCC specimens, and were compared with corresponding normal tissues. Before the cDNA synthesis, residual genomic DNA was removed from the total RNA by

Table I. Profiles of OSCC cases.

Factors	Data
Gender	
Male	26
Female	13
Age	
Min	27 years
Max	87 years
Average	64.4 years
SD	13.1
Lesion sites	
Tongue	20
Gingiva	8
Buccal mucosa	4
Oral floor	3
Fauces	2
Lip	1
Mandibular bone	1
Differentiation	
Well	23
Moderately	15
Poorly	1
Primary tumor size	
T1	1
T2	11
T3	17
T4	10
Regional lymph node metastasis	
N0	18
N1	8
N2	15
Distant metastasis	
No	27
Unknown	12
Stage	
I	1
II	7
III	10
IV	21
pN[a]	
No	22
Yes	17

[a]pN, Pathologically diagnosed regional lymph node metastasis.

DNase I treatment (DNA-free kit; Ambion, Austin, TX, USA). The nucleotide sequences of the gene-specific primers for qRT-PCR amplification of *IL1RN* were: forward 5'-TCCAAG CTCCATCTCCACTC-3', reverse 5'-GCTGAGTACCTGC
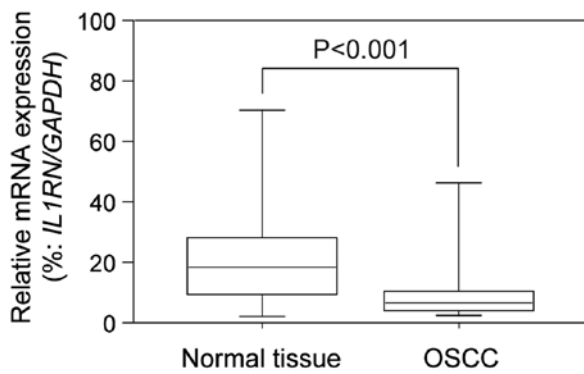
Figure 1. *IL1RN* mRNA expression in OSCC samples and corresponding normal tissues. qRT-PCR performed using RNA from OSCC samples and corresponding normal tissues, was applied to examine differences in the expression levels of *IL1RN* mRNA between the two tissues. The results are expressed as the means ± SD, and analyzed using unpaired Student's t-test.

CAAGAGC-3'. The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH; forward 5'-TTGGTATCGTGGA AGGACTGA-3', reverse 5'-TGTCATGATATTTGGCAG GTTT-3') was used as an internal control. The sequences of specific primers were checked before use by the Primer3 program (available at http:www-genome.wi.mit.edu/cgi-bin/ primer/primer3_www.cgi), to avoid amplification of genomic DNA or pseudogenes. Amplified products were analyzed by 3% agarose gel electrophoresis to ascertain their size and purity.

Real-time qRT-PCR was performed using the LightCycler FastStart DNA Master SYBR Green I kit (Roche, Indianapolis, IN, USA). To prepare the standard curve, 3 μg of total RNA from normal oral tissue was reverse-transcribed with Superscript RT (Life Technologies) and oligo-d(T)12-18 primer, after which, serial dilutions were made corresponding to cDNA transcribed from 300, 30, 3.0 and 0.3 ng of total RNA. PCRs using the LightCycler apparatus were carried out in a final volume of 20 μl of reaction mixture, consisting of 2 μl of FirstStart DNA Master SYBR Green I mix, 3 mM $MgCl_2$ and 0.2 μl of primers, according to the manufacturer's instructions. The reaction mixture was then loaded into glass capillary tubes. For real-time qRT-PCR, the DNA was subjected to an initial denaturation at 95˚C for 10 min, followed by 45 rounds of amplification at 95˚C (10 sec) for denaturation, 58˚C (10 sec) for annealing and 72˚C for extension, with a temperature slope of 20˚C/sec. The transcript levels of *IL1RN* were estimated from the respective standard curves and normalized to the *GAPDH* transcript in corresponding samples.

Immunohistochemistry (IHC). Immunohistochemical staining was performed using a series of OSCC specimens, which included 39 OSCCs with corresponding normal tissues, and 18 OPLs that were diagnosed histopathologically as leukoplakia with epithelial dysplasia. Considering that evidence has shown that the malignant transformation rate of oral leukoplakia with dysplasia is higher than that of oral leukoplakia without dysplasia (14), patients with advanced OPLs, defined as leukoplakia exhibiting epithelial dysplasia, were considered to be eligible for this study. IHC staining was carried out on 4 μm sections of paraffin-embedded specimens. Briefly, after deparaffinization and hydration, the slides were pretreated in

10 mM sodium citrate buffer (pH 6.0) in a microwave oven for 5 min at 95˚C.

The endogenous peroxidase activity was quenched by a 30-min incubation in a mixture of 0.3% hydrogen peroxide solution in 100% methanol. After washing with PBS buffer, the sections then were incubated with the primary antibody (biotinylated goat anti-human IL1RN antibody (1:100 dilution; R&D Systems, Inc., Minneapolis, MN, USA) at room temperature and in a humidified chamber for 2 h. After washing with PBS buffer, the slides were treated with peroxidase-labeled secondary antibody for 1 h, followed by color development in 3,3'-diaminobenzidine tetrahydrochloride (Dako Japan Inc.). Finally, the slides were lightly counterstained with hematoxylin. As negative controls, slides were incubated with PBS instead of the primary antibody. To quantify the IL1RN protein expression, we used the Histo-score (H-score) system as previously described (15,16). In brief, the mean percentage of epithelial cells exhibiting a persistent IL1RN signal was determined in at least five distinct fields using x400 magnification in each section. The intensity of the immunoreaction was scored as follows: 1+, weak; 2+, moderate and 3+, intense. Three target cell types: normal, premalignant and malignant epithelial cells, were identified for scoring.

In the present study, IL1RN immunoreactivity was detected mainly on the plasma membrane of target cells, and heterogeneously in the cytoplasm. The plasma membrane IL1RN immunoreaction was selected for the scoring. The percentage of IL1RN-positive cells and the staining intensity were then multiplied to produce the IL1RN H-score. The H-scoring of each sample was performed by two independent pathologists, neither of whom had knowledge of the clinical status of the patients.

Statistical analysis. The statistical analyses of the expression levels of IL1RN mRNA and *IL1RN* protein were performed using unpaired or paired Student's t-test. P<0.05 was considered to be statistically significant.

Results

IL1RN mRNA expression in OSCC tissues. According to real-time qRT-PCR data, the *IL1RN* mRNA expression in OSCC specimens was significantly (P<0.001) lower than that of the corresponding normal mucosa (Fig. 1). The relative *IL1RN* mRNA expression levels in normal and tumor sites ranged from 0.022 to 4.616 (median, 0.397) and 0.050 to 8.565 (median, 1.335), respectively.

IL1RN expression in OSCCs and OPLs. Representative results for IL1RN protein expression in normal oral tissues, OPLs and primary OSCCs are shown in Fig. 2. Normal oral mucosal specimens exhibited consistently strong IL1RN immunoreactivity on the plasma membrane of cells. According to the H-scores, the expression levels of plasma membranous IL1RN were significantly reduced not only in OSCCs, but also in OPLs, in comparison to those of normal tissues (Fig. 3). In OPLs, although a case with mild dysplasia showed positive plasma membranous immunostaining for IL1RN, the staining signals decreased gradually in moderate through to severe dysplasia
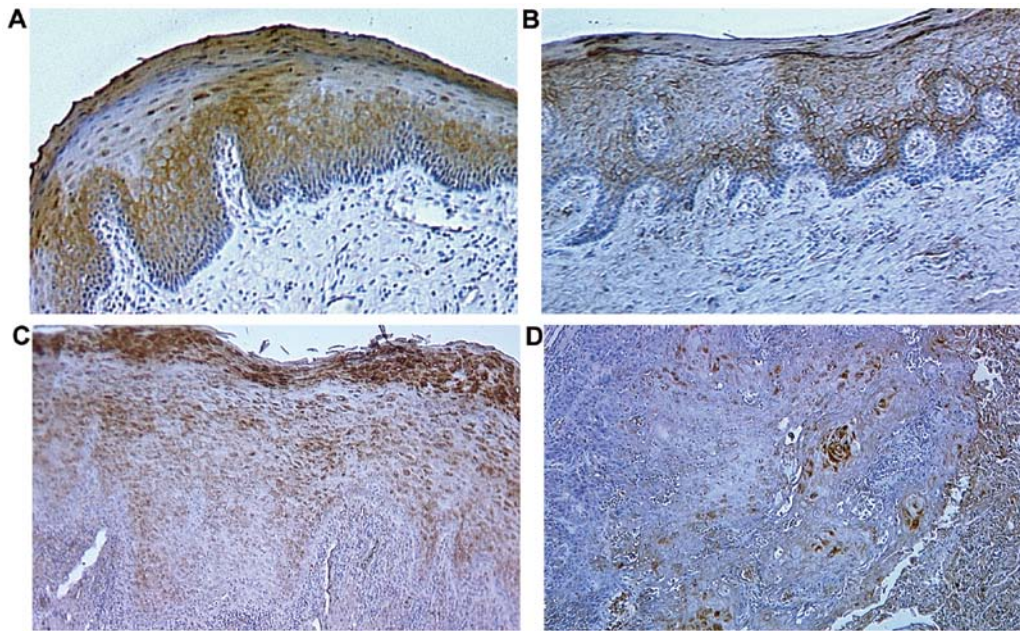
Figure 2. Representative results of immunohistochemical staining for IL1RN in normal tissues, OPLs and OSCCs. IHC staining of tissue sections shows IL1RN in brown. (A) Normal oral tissue. (B) OPL with mild dysplasia. (C) OPL with severe dysplasia. (D) OSCC. Original magnification was x200.
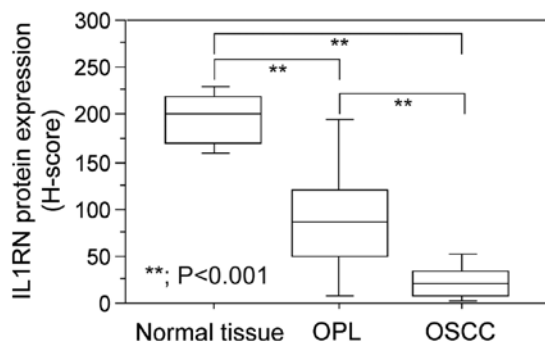

Figure 3. H-scores for the plasma membranous IL1RN expression in normal tissues, OPLs and OSCCs. To determine the extent of IL1RN expression across normal tissues, OPLs and OSCCs, H-scoring was performed on IL1RN stained tissues as described in Materials and methods. The results are expressed as the mean H-score ± SD for each tissue type, and analyzed using unpaired Student's t-test.
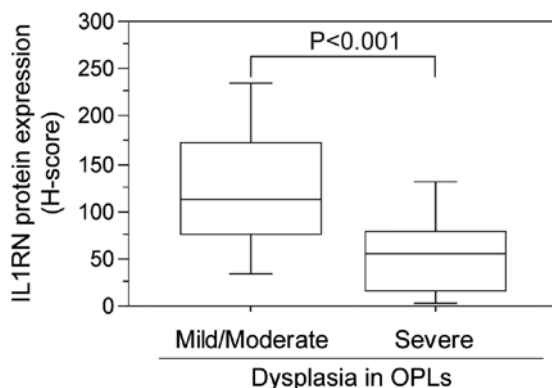

Figure 4. Correlation between IL1RN expression and the dysplasia grade in OPLs. Differences in IL1RN expression according to dysplasia grade in OPLs, i.e., mild/moderate dysplasia versus severe dysplasia, were determined by H-scoring of IL1RN stained tissues. The results are expressed as the mean H-score ± SD for each tissue type, and analyzed using unpaired Student's t-test.

cases. Significant differences were observed between mild/moderate dysplasia cases and severe dysplasia cases (Fig. 4). Notably, IL1RN staining was also detected heterogeneously in the cytoplasm of OPL cells in some cases.

In OSCCs, the IL1RN immunoreactivity was largely lost in the specimens examined. Significantly downregulated IL1RN expression was observed in all lesion sites examined when compared with the matched normal tissues (P<0.001) (Fig. 5). However, the decreased level of IL1RN expression did not correspond with tumor progression (Fig. 6). Relative IL1RN expression levels, determined as relative values of H-scores in tumor samples compared with normal tissues, were analyzed alongside clinicopathological factors. Noteworthy, IL1RN expression was higher in the advanced OSCC cases (T3/T4) in comparison to the early cases (T1/T2) (P=0.0275) (Fig. 6A). Significant alteration of IL1RN associated with regional lymph node metastasis (Fig. 6B), TNM staging (Fig. 6C), differentiation (Fig. 6D) was not observed. OSCC cases were divided into two groups according to lesion sites: buccal mucosa/oral floor/fauces/gingival, and tongue. IL1RN expression associated with the clinicopathological factors was also analyzed separately, and the data revealed that higher IL1RN expression was associated with active tumor development: tumor size (Fig. 7A), regional lymph node metastasis (Fig. 7B), TNM staging (Fig. 7C), in the OSCC in buccal mucosa, oral floor, fauces and gingiva but not in the tongue. No significant difference was found between well and moderately/poorly differentiated OSCCs (Fig. 7D).

Discussion

A real-time qRT-PCR assay revealed that the relative *IL1RN* mRNA expression levels were significantly lower in OSCCs than in their normal counterparts, which agrees with the recent study by Lallemant *et al* (17). Similarly, the IL1RN expression was significantly downregulated in the OSCC
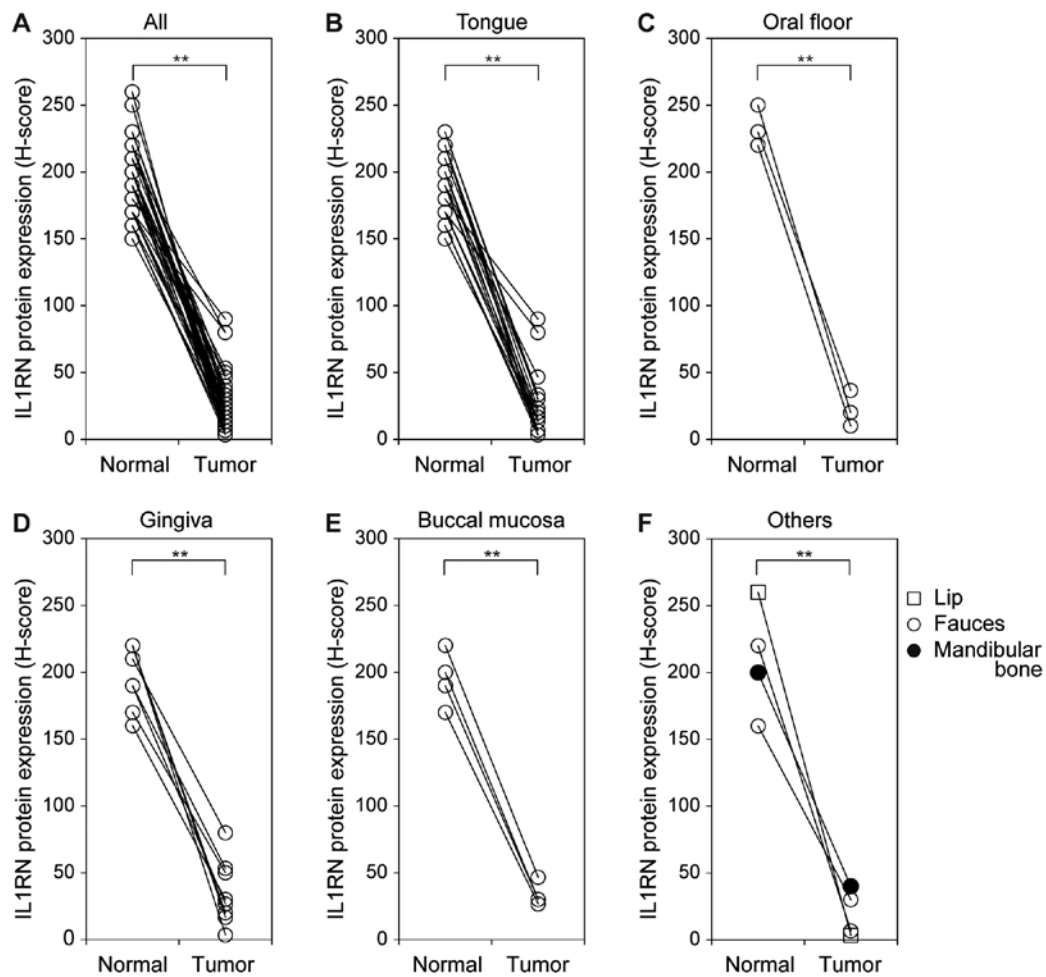
Figure 5. Comparison of IL1RN expression between OSCCs and the matched samples in each lesion site. IHC staining for IL1RN, and subsequent H-scoring, was performed on OSCCs (T) and the normal matched samples (N). H-scores for the matched N and T samples are plotted according to the lesion site from which they were acquired. Significant differences between the N and T of H-scores for each lesion site, and across all samples (Total), are indicated (P<0.001, paired Student's t-test).
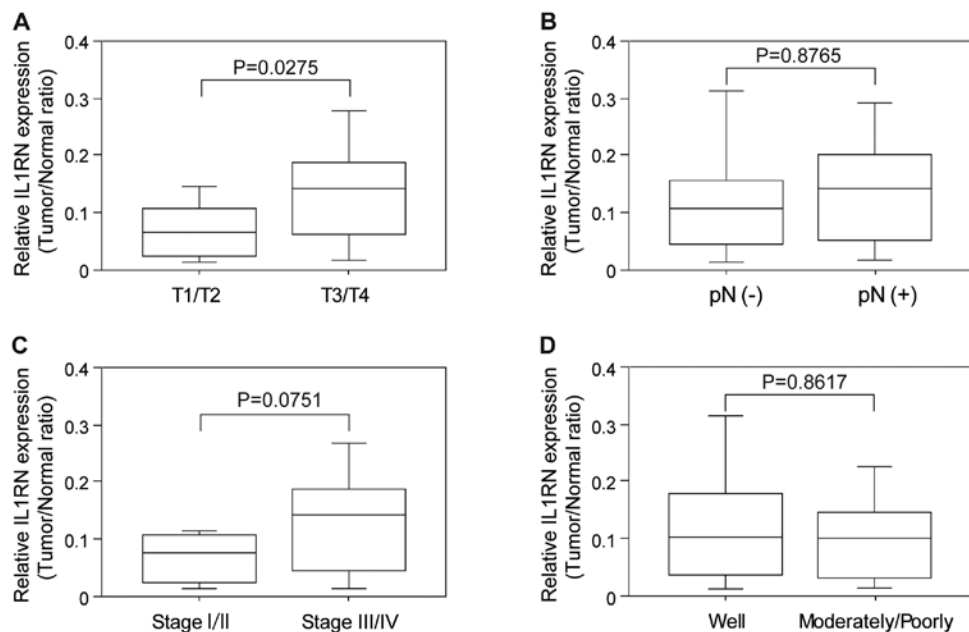

Figure 6. Analysis of IL1RN expression according to clinicopathological factors in OSCC cases. IL1RN expression levels in OSCCs determined as relative values of H-scores in tumor samples compared with normal tissues, were further evaluated according to the following clinicopathological factors: primary tumor size (A), staging (B), pathologically determined metastasis of regional lymph nodes (C), differentiation (D). IL1RN expression was higher in the advanced OSCC cases (T3/T4) than the early cases (T1/T2). The results are expressed as the mean relative H-score ± SD, and analyzed using unpaired Student's t-test.
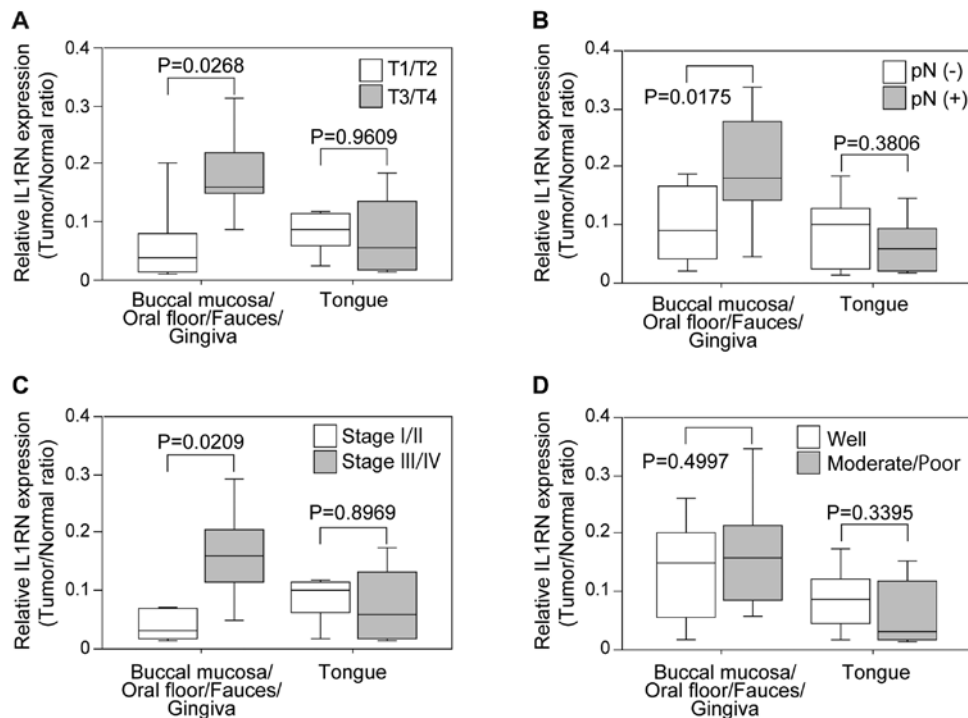
Figure 7. Analysis of lesion site-dependent IL1RN expression, associated with clinicopathological factors in OSCCs. Lesion site-dependent IL1RN expression levels determined as relative values of H-scores in tumor samples compared with normal tissues, were further analyzed according to primary tumor size (A), staging (B), pathologically determined metastasis of regional lymph nodes (C), differentiation (D). In OSCC cases originated from buccal mucosa/oral floor/fauces/gingiva advanced primary tumor size, staging and pathologically detected metastasis were closely associated with upregulated expression of IL1RN. However, no significant correlation between IL1RN expression and clinicopathological factors was found in tongue OSCC cases. The results are expressed as the mean relative H-score ± SD, and analyzed using unpaired Student's t-test.

specimens examined compared to the normal oral epithelium. Yamamoto *et al* showed that in OSCCs, a significant loss of heterozygosity occurred at alleles in chromosome band 2q14, where the IL1RN gene is located (18), and that genetic variations of IL1RN were strongly associated with the IL1RN expression level (19,20). This suggested that altered IL1RN expression at the mRNA and protein levels could result from gene mutations in OSCC.

Low levels of IL1RN have been associated with greater disease severity in a variety of human malignancies, including leukemia (21), myeloma (22), colorectal cancer (23) and prostate cancer (24). In addition, experimental studies have shown that IL1RN decreases tumor growth or aggressive behavior by inhibiting IL-1-mediated activities in the cancer cells. Enhanced expression of IL1RN inhibits tumor growth of skin carcinoma cells *in vitro* and *in vivo* through blocking the transcriptional effect of IL-1 on cyclooxygenase-2, which is thought to play a pivotal role in tumor development (25). Elaraj *et al* showed that recombinant IL1RN significantly inhibited the tumor growth and metastatic potential of a human melanoma xenograft that constitutively secreted IL-1 (26). They also detected that IL1RN had anti-angiogenic effects in tumors, where it decreased the IL-1-mediated induction of angiogenic molecules, including interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF). Similar findings were also observed for human breast cancer (27). These findings and our present results indicate that IL1RN may exhibit tumor-suppressing activity.

In contrast, there have been some contradictory observations that suggest IL1RN could increase tumorigenesis. For

example, IL1RN was significantly overexpressed in cervical carcinomas compared with their normal counterparts (28). Additionally, IL1RN upregulation was also detected in gastric cancer, and was associated with a high incidence of metastases (29). Furthermore, *in vitro* studies showed that IL1RN enhances the growth and proliferation of human glioblastoma (30), prostatic (31) and hepatic cancer cells (32). Considering that IL-1 plays multiple biological roles in various tissues, the effect of IL1RN may be cell or tissue type-specific, or microenvironment-specific; for example, it may enhance cytotoxic T cell activity, and the tumoricidal capacities of natural killer (NK) cells (33,34), and alterations in the properties of IL1RN are likely due to changes in the local IL-1-dependent pathways. Although the details of the biological mechanisms responsible for IL1RN alterations in tumors have not been determined, our results suggest that IL1RN may be critical for maintaining the normal condition of cells, and that a loss of the expression of the protein could result in an enhanced risk of cancer development in the oral cavity.

The data described in the present study also showed that the IL1RN expression was gradually downregulated in accordance with the degree of dysplasia. As oral dysplasia shows a significant rate of transformation to cancer (35,36), there is an urgent need to identify better ways to predict which patients with dysplastic precursor lesions will develop OSCC. The present results suggest that IL1RN may suppress the early carcinogenic events and that the expression level could represent a useful biomarker for the early diagnosis of OSCC. However, our data revealed that relatively higher expression of

IL1RN is found in the developed OSCC samples, especially in the OSCC occurring in buccal mucosa, oral floor, fauces and gingiva but not in the tongue.

In conclusion, IL1RN may exhibit conflicting characteristics in controlling oral malignancies; namely, suppressive activity in the early carcinogenic events, but also lesion site-dependent tumor development promotion. Thus, IL1RN should be a reliable biomarker for the early diagnosis of OSCC. However, IL1RN may have unknown and complicated functions in the developed OSCC, suggesting that further investigation is necessary before considering IL1RN as a therapeutic target in oral cancer.

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