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 (I). 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
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 ventrolateral 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 pathological 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 transcription, 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 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'-TCCAAGCTCCATCTCCACTC-3', reverse 5'-GCTGAGTACCTGC
of amplification at 95˚C (10 sec) for denaturation, 58˚C (10 sec)

- The reaction mixture was then loaded into glass capillary
- 0.2 µl of primers, according to the manufacturer's instructions.

CAAGAGC-3'. The glyceraldehyde-3-phosphate dehydro-

- expression levels of corresponding normal tissues, was applied to examine differences in the
- OSCC samples and corresponding normal tissues. qRT-PCR performed using RNA from OSCC samples and
- normal tissues. qRT-PCR performed using RNA from OSCC samples and corresponding normal tissues. The results are
- the means ± SD, and analyzed using unpaired Student’s t-test.

Real-time qRT-PCR was performed using the LightCycler
- FastStart DNA Master SYBR Green I mix, 3 mM MgCl
- primer, after which, serial dilutions were made corresponding
to cDNA transcribed from 20˚C/sec. The transcript levels of
- were estimated to be eligible for this study. IHC staining was carried out on
- to determine the IL1RN H-score. The
- reached moderate staining intensity. Cells with weak
- intensity of the immunoreaction was scored as follows: 1+,
- increased gradually in moderate through to severe dysplasia.
- in OSCCs were significantly (P<0.001) lower than that
- by color development in 3,3'-diaminobenzidine tetrahydro-
- was then multiplied to produce the IL1RN H-score. The
- normal, premalignant and malignant epithelial cells, were
- intensity; R&D Systems, Inc., Minneapolis, MN, USA) at room
- 3 min, followed by a 10-min incubation with 100 mM
- and in OSCC tissues. A persistent IL1RN signal was determined in at least five
- a persistent IL1RN signal was determined in at least five
- to 0.0001) lower than that of the corresponding normal mucosa (Fig. 1). The relative
- for IL1RN protein expression in normal oral tissues, OPLs and
- OSCC specimens was significantly (P<0.001) lower than that
- and in OSCC tissues. A persistent IL1RN signal was determined in at least five
- IL1RN immunoreactivity was detected mainly on the plasma membrane of target cells, and
- cytoplasm. The plasma membrane IL1RN immunoreaction was selected for the scoring. The
- three target cell types: normal, premalignant and malignant epithelial cells, were
- sections using x400 magnification in each section. The
- 5 min at 95˚C.

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 dehydro-
genase gene (GAPDH; forward 5'-TTGTTATCGTGGA
AGGACTGA-3’, reverse 5'-TGTGATATGATTGGCA
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.
PCR amplification was performed in a
- Real-time qRT-PCR using the LightCycler apparatus were carried out in a
- 10 mM sodium citrate buffer (pH 6.0) in a microwave oven for
- The endogenous peroxidase activity was quenched by a
- room temperature and in a humidified chamber for 2 h. After
- the slides were incubated with the primary antibody (biotinylated goat anti-human IL1RN antibody (1:100 dilution;
R&D Systems, Inc., Minneapolis, MN, USA) at room
- to the plasma membrane of target cells. According to the
-H-scores, the expression levels of plasma membranous IL1RN
expression levels in OSCC and OPLs.

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 leuko-
-plakia with epithelial dysplasia. Considering that evidence has
shown that the malignant transformation rate of oral leuko-
-plakia 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
- temperature and in a humidified chamber for 2 h. After
- The percentage of IL1RN-positive cells and the staining intensity
- The intensity of the immunoreaction was scored as follows: 1+, weak; 2+, moderate and 3+, intense. Three target cell types:
- IL1RN expression in OSCCs and OPLs.

Representative results

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 immunono-
reactivity 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
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/gingiva, 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 cases.
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.
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.

References