Original article

Expression of osteoclast-related cytokines in mandibular invasion by gingival squamous cell carcinoma

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A B S T R A C T

Gingival squamous cell carcinoma (GSCC) displays strong potential for local infiltration. Detailed evaluation of bone invasion to the mandible is an important problem clinically. The purpose of this study was to investigate the expression of osteoclast-related cytokines, which play an important role in mandibular invasion. A total of 23 cases of lower GSCC were classified histopathologically as non-invasive group (n = 10) or invasive group (n = 13). Cytokines of interleukin (IL)-1α, IL-1β, IL-6, IL-11, tumor necrosis factor (TNF-α), parathyroid hormone-related protein (PTHrP), receptor activator of nuclear factor κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) were selected. In this prospective study, the expressions of these cytokines were studied by quantitative real-time reverse transcription-polymerase chain reaction. IL-1α, IL-1β, IL-6, TNF-α, PTHrP, and RANKL were expressed higher in the invasive group. In particular, IL-6 was considered as a non-specific cytokine which responds to inflammatory change by tumor or non-tumor stimulation. These results suggest that IL-1α, IL-1β, IL-6, TNF-α, PTHrP, and RANKL play important roles in the mechanisms underlying mandibular invasion. Furthermore, expression analysis of OPG/RANK/RANKL system may contribute to treatment and prevention of GSCC in future.

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1. Introduction

Gingival squamous cell carcinoma (GSCC) is a malignant tumor that frequency invades the maxilla and mandibular bone anatomically [1,2]. Clinically, detailed evaluation of mandibular invasion is important when deciding whether to perform mandibulectomy associated with deterioration in the quality of life [3–5]. The extent of mandibular invasion was only assessed by radiography such as computed tomography (CT) and magnetic resonance imaging. However they do not always make a precise diagnosis [6–11].

In several types of cancers, numerous studies have investigated the functional mechanisms of osteoblasts and osteoclasts with various cytokines related to bone metastasis [1,2–14]. They also have focused on histopathological bone invasion caused by activation of osteoclast by osteoclast-related cytokines including interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-11 (IL-11), tumor necrosis factor-α (TNF-α), and parathyroid hormone-related protein (PTHrP) [15,16]. Furthermore they have reported that osteoblasts might act on pre-osteoclasts and differentiation to osteoclasts is promoted by a pathway of the osteoprotegerin (OPG)/receptor activator of nuclear factor κB (RANK)/RANK ligand (RANKL) system in vitro [17,18]. Although it has been reported that the RANKL/RANK signaling pathway also regulates bone invasion or metastasis in oral squamous cell carcinoma (OSCC) cell lines, it remains unclear for human OSCC [18]. In addition, most reports have used a histopathological approach in the clinical study design for the expression of those cytokines [1,9–11,16].

The purpose of this study was to investigate the network system of osteoclast-related cytokines in a prospective study, which play important roles in mandibular invasion by human GSCC.

2. Materials and methods

In this prospective study, the subjects were 23 patients with lower GSCC who underwent mandibulectomy at the Department of Oral and Maxillofacial Surgery, Tokyo Dental College between July 2004 and June 2007. Mean age (±S.D.) was 65.0 (±8.6) years. There were 14 males and 9 females. Informed consent was obtained from each patient according to a protocol that was reviewed and approved by the ethical review board of Tokyo Dental College.

2.1. Clinicopathological analysis of mandibular invasion

According to resection specimens, which were stained using hematoxylin and eosin, tumors were classified histopathologically into two groups as we have reported [16]. In the non-invasive group, diffuse tumor cells were apparent near the mandible...
without invasion into bone. Fibrous connective tissue was seen at the border between tumor and bone. In the erosive type, tumor cells were histopathologically identified directly adjacent to the bone surface, although the bone surface was clear, smooth and continuous despite absence of an intervening layer of connective tissue. The invasive group, was defined as Brown's infiltrative type with tumor cells invading and infiltrating into bone and osteoclasts appearing along the bone surface [14,19,20]. Tumor cells advanced independently into cancellous bone without an intervening layer of connective tissue (Fig. 1).

2.2. mRNA expression analysis

The specimens were taken from the center of tumors at the time of surgical resection. After resection, each sample was immediately kept at −80°C until analyzed. Total RNA samples were prepared using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), according to the protocols of the manufacturer. For gene expression of 9 cytokines by quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR), 2 μg of total RNA was reverse-transcribed into cDNA using a one-step kit (ready-to-go RT-PCR Beads; GE Healthcare, Buckinghamshire, UK) and oligo (dT)12–18 primer (Invitrogen).

QRT-PCR using the TaqMan MGB probe (Applied Biosystems, Foster City, CA, USA) was performed with modifications to the manufacturer’s instructions [21–23]. GAPDH was used as an internal control (Cat. No. 4333764F; Applied Biosystems). TaqMan MGB probes of IL-1α, IL-1β, IL-6, IL-11, TNF-α, PTHrP, RANK, RANKL, and OPG were purchased from Applied Biosystems (Assay ID: Hs00174092_m1, Hs00174097_m1, Hs00174131_m1, Hs00174148_m1, Hs00174128_m1, Hs00174969_m1, Hs00187189_m1, Hs00243522_m1, and Hs00171068_m1, respectively). PCR reactions using the primer sets in an ABI PRISM 7700 Sequence Detector (Applied Biosystems) were performed in a final volume of 25 μl of reaction mixture: 2 μl of previously diluted cDNA, followed by addition of 1 μl of 20× TaqMan primers and probe and 10 μl of TaqMan Universal Master Mix (Applied Biosystems), according to the manufacturer’s instructions. An initial denaturation step was performed at 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15 s and annealing or extension at 60°C for 1 min. ABI7700 system software was used to monitor in FAM dye. Threshold cycle (Ct) value for each reaction reflects the amount of PCR needed to identify a target gene, and the relative level of 9 cytokines for each sample was calculated as outlined by the manufacturer. GAPDH was used to normalize amounts of cytokine mRNA. The Ct value was subtracted from that of the cytokine gene to obtain a ΔCt value. The difference (ΔΔCt) between ΔCt of each sample for the gene target and ΔCt of the calibrator was determined. Specimens were analyzed 3 times and values were averaged.

2.3. Immunohistochemical staining

The cases of mRNA expression were studied by immunohistochemical analysis. Selection samples were decalcified, paraffinized, and sectioned at 4 μm thick. Sections were deparaffinized in xylene, hydrated in graded ethanol, and then heated in the microwave in 0.01 M sodium citrate solution at 60°C for 20 min. To eliminate endogenous peroxidase, sections were immersed in 3% H2O2 solution in methanol at room temperature for 15 min. Sections were washed with phosphate-buffered saline (PBS; pH 7.4) for 15 min. Non-specific reactions were blocked by treatment with 10% normal rabbit serum at room temperature for 30 min. Sections were incubated with primary antibodies diluted at 4°C overnight. Sections were then treated using secondary antibodies (biotin-labeled mouse IgG, IgA, and IgM antibodies for IL-1α, IL-1β, PTHrP, RANK, RANKL, and OPG and biotin-labeled goat antibodies for IL-6, IL-11, and TNF-α) at room temperature for 30 min. Thereafter, sections were immersed in peroxidase-labeled streptavidin at room temperature for 30 min (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan). Color reaction was performed using 3,3-diaminobenzidine-4-hydrochloride Tris–HCl buffer containing 0.05% H2O2 solution for 5 min. Mayer’s comparative staining with hematoxylin was performed, and specimens were examined under microscopy at ×400.

Specimens treated with PBS alone instead of with the primary antibodies were used as negative controls, and specimens with more pronounced staining reaction in the cytoplasm than negative controls were classed as antibody-positive.

2.4. Statistical analysis

Mann–Whitney’s U-test and Chi-square test were performed to evaluate correlations between the non-invasive and the invasive groups. Values of p < 0.05 were considered statistically significant.

3. Results

3.1. Clinicopathological analysis

From the resection specimens of these cases, tumors were grouped by microscopic examination into 10 cases in the non-invasive group and 13 cases in the invasive group. Patients
Table 1

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primary antibody</th>
<th>Marker</th>
<th>Dilution ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1alpha</td>
<td>Rabbit anti-human IL-1α polyclonal antibody</td>
<td>ENDOGEN</td>
<td>1/100</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Rabbit anti-human IL-1β polyclonal antibody</td>
<td>ENDOGEN</td>
<td>1/100</td>
</tr>
<tr>
<td>IL-6</td>
<td>Goat anti-human IL-6 polyclonal antibody</td>
<td>Santa Cruz Biotechnology</td>
<td>1/100</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>Goat anti-human TNF-α polyclonal antibody</td>
<td>R&amp;D Systems</td>
<td>1/100</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Mouse anti-human PTHrP monoclonal antibody</td>
<td>CALBIOCHEM</td>
<td>1/100</td>
</tr>
<tr>
<td>RANKL</td>
<td>Mouse anti-human RANKL monoclonal antibody</td>
<td>R&amp;D Systems</td>
<td>1/100</td>
</tr>
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</table>

Comprised 6 males and 4 females in the non-invasive group, and 8 males and 5 females in the invasive group. Mean age (±S.D.) was 62.8 (±8.7) years in the non-invasive group and 66.7 (±8.4) years in the invasive group. According to Union for International Cancer Control classifications [19], the non-invasive group was T1–T2 in 5 cases and T3–T4 in 5 cases. In contrast, the invasive group was T1–T4 in 5 cases and T3–T4 in 8 cases. Furthermore, N positive cases consisted of 8 cases in the non-invasive group and 11 cases in the invasive group. According to tumor differentiation, the non-invasive group included 4 cases with mildly differentiated, 4 cases with moderately differentiated, and 2 cases with poorly differentiated cells. In contrast, the invasive group included 6 cases with mildly differentiated and 7 cases with moderately differentiated cells. For all clinical data, there were no significant differences between the non-invasive group and the invasive group by Mann–Whitney’s U-test and Chi-square test (Table 1).

3.2. mRNA expression analysis

In the non-invasive group, mean mRNA expression levels (Ct value) were 0.07 (range, 0.0–0.33) for IL-1α, 0.34 (range, 0.0006–2.46) for IL-1β, 117.93 (range, 0.55–519.15) for IL-6, 17.70 (range, 8.54–32.75) for TNF-α, 1.09 (range, 0.08–4.05) for PTHrP, and 16.44 (range, 2.49–34.66) for RANKL. In the invasive group, mean mRNA expression was 14.78 (range, 0.002–161.27) for IL-1α, 41.68 (range, 0.04–492.28) for IL-1β, 612.28 (range, 3.14–6137.07) for IL-6, 31.58 (range, 1.00–174.65) for TNF-α, 4.74 (range, 0.09–21.96) for PTHrP, and 58.01 (range, 1.01–278.20) for RANKL [IL-1α (p = 0.277), IL-1β (p = 0.099), IL-6 (p = 0.849), TNF-α (p = 0.425), PTHrP (p = 0.086), and RANKL (p = 0.558)]. Though these expressions were not significant, the mRNA expression of IL-1α, IL-1β, IL-6, TNF-α, PTHrP, and RANKL was higher in the invasive group than in the non-invasive group. In particular, the expression of IL-6 was higher. IL-11, RANK, and OPG were not expressed (Fig. 2).

3.3. Immunohistochemical staining

We observed immunohistochemical staining for 6 cytokines including IL-1α, IL-1β, IL-6, TNF-α, PTHrP, and RANKL because of the high values of mRNA expression in the invasive group. Six antibodies were used in some cases of each group (Table 2). There was more positive staining in cells for IL-1α, IL-1β, IL-6, TNF-α, PTHrP, and RANKL in the invasive cases than in the non-invasive cases (Fig. 3). In each specimen, positive cells of these cytokines included: 6

**Fig. 2.** Expression of 5 cytokines related to osteoclasts in resection specimens by real-time quantitative polymerase chain reaction. Results were compared between non-invasive and invasive groups. Y-axis means the threshold cycle (Ct) value difference between that of each cytokine gene and that of the calibrator by ABI7700 system software. Values of p < 0.05 were considered significant (Mann–Whitney’s U-test), IL: interleukin; TNF, tumor necrosis factor; PTHrP, parathyroid hormone-related protein; RANK, receptor activator of nuclear factor κB; RANKL, RANK ligand; OPG, osteoprotegerin. □: non-invasive group; ■: invasive group; NE: no expression.
were observed on not only bone surface but also distant from bone. In the invasive group an increased number of osteoclasts at the tumor/bone interface was mostly observed microscopically (Figs. 1 and 3). Most immunohistochemical staining revealed positive-stained cells in the specimens as follows: IL-1α, lymphocyte; IL-1β, IL-6, and TNF-α, lymphocyte and tumor cell; PTHrP, tumor cells; RANKL, stromal cell-like cell. The expressions of these cytokines revealed both positive-stained cytoplasm and nuclei in the tumor cells.

4. Discussion

Bone resorption by osteoclasts is an important step in the process of bone invasion and bone metastasis for several types of human cancers including renal cancer, breast cancer, and multiple myeloma [22,23]. Furthermore, we have previously shown that human OSCC causing mandibular invasion was also found to release osteoclast-related cytokines including IL-6, IL-11, TNF-α, and PTHrP [16]. From clinical data in this study, there were no clear correlations between differentiation type which showed general evaluation of malignancy and mandibular invasion type in human GSCC. As a result, the present study focused attention on cytokines that reportedly contribute to bone invasion and bone metastasis for several types of cancers [15,16,22,23]: IL-1α, IL-1β, IL-6, IL-11, TNF-α, PTHrP, RANK, RANKL, and OPG. In QRT-PCR, levels of IL-1α, IL-1β, IL-6, TNF-α, PTHrP, and RANKL mRNA were clearly, but non-significantly, higher in the invasive group. Moreover, the mRNA expression level of IL-6 appeared greatly higher in the invasive group. In the meantime IL-6 mRNA expression level varied greatly in each case and the range was wider. This is because IL-6 is a multifunctional cytokine including inflammatory change with a strong nonspecific reaction and is affected by various mechanisms, unlike the other cytokines [13,15]. In vitro, some human OSCC cell lines are known to produce osteoclast-related cytokines including inflammatory cytokines such as IL-1, IL-1β, and IL-6 [15,18,24]. In comparison, PTHrP and RANKL are known to be activated specifically in pre-osteoclasts and osteoclasts [18,25,26]. For IL-11, RANK, and OPG, no expression was identified by QRT-PCR. On the other hand, there are some reports indicating that IL-11, RANK, and OPG are important in the process of bone invasion in several cancers. It was also reported that positive rate of immunohistochemical staining for RANK was not significantly higher in bone-invasive OSCC than in non-invasive OSCC. IL-11, RANK, and OPG may not contribute to the mandibular invasion of GSCC. Furthermore, in bone invasive OSCC, the positive rate of immunohistochemical staining for RANK was not significantly higher than in non-invasive OSCC [27]. Our study is also similar. However, it is not clear about the difference between OSCC and the other cancers. Even if no clear significant difference was found in expression, IL-1α, IL-1β, IL-6, TNF-α, PTHrP, and RANKL may play important roles in the mechanism of mandibular invasion by GSCC. Generally the pathways of these cytokines are very complex. IL-1α and IL-1β have been reported as playing a prominent role in enhancing transcription and expression of inflammatory cytokines such as IL-6 [24,25]. IL-1α, IL-6, TNF-α, and PTHrP have been shown to activate osteoclasts and PTHrP activates osteoblasts to up-regulate RANKL expression [18,26]. RANKL then binds to RANK expressed in pre-osteoclasts and promotes differentiation to mature osteoclasts [17,18]. This cycle results in the development and progression of bone invasion and bone metastasis. OPG inhibits the differentiation and activity of osteoclasts, which is a decoy receptor of RANKL. In this study, PTHrP and RANKL might be the starting point of the OPG/RANK/RANKL system expressed in a bone resorption process. IL-1α, IL-1β, IL-6,
and TNF-α might reinforce activation of osteoclasts by this system. Consequently, inhibition of RANK/RANKL system is expected to be a therapeutically target to prevent bone invasion by GSCC. The locality of these cytokines was also the subject of attention. Positive cells for each cytokine were observed both on the bone surface and distant from bone by immunohistochemical staining. Expression of these cytokines thus did not seem to depend on the locus, and cytokines were delivered directly from OSCC cells. Potentially, even small biopsy samples of GSCC may allow evaluation of the degree of bone invasion according to expression of these cytokines.

In conclusion, our study suggests that human GSCC cells released IL-1α, IL-1β, IL-6, TNF-α, PTHrP, and RANKL, which are osteoclast-related cytokines and activity of the OPG/RANK/RANKL system finally caused mandibular invasion. Understanding the mechanisms of the cytokine network in detail will be necessary to contribute to treatment and prevention of bone invasion by GSCC in the future.

Conflict of interest

We declare no conflict of interest.

Acknowledgments

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