**Prognostic significance of CD44v3 and CD44v6 in oral squamous cell carcinoma of the tongue**

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**Abstract**

Background: CD44, a family of alternatively spliced transmembrane cell adhesion molecules, is expressed in many types of tumors, including oral squamous cell carcinoma. CD44 variant 3 and 6 (CD44v3 and CD44v6) expression in head and neck squamous cell carcinoma has been suggested to correlate with a poor prognosis. We investigated the relationship between CD44 variant forms and clinicopathological parameters in oral squamous cell carcinoma of the tongue (OSCCT).

Methods: Immunohistochemical analysis of 60 cases of OSCCT was performed to investigate the relationship of CD44v3 and CD44v6 expression with clinicopathological parameters and clinical outcome.

Results: Significant correlations were present between CD44v3 expression and clinicopathological parameters, such as mode of invasion ($P<0.0001$), differentiation type ($P<0.0001$), local recurrence ($P<0.05$), and secondary metastasis to lymph nodes ($P<0.05$). Loss of staining of CD44v3 also significantly correlated with a poor prognosis ($P<0.001$). On the other hand, CD44v6 expression showed no correlation with clinicopathological parameters. Conclusion: CD44v3 may be useful as a diagnostic/prognostic biomarker of OSCCT because low CD44v3 immunoreactivity is associated with high invasive potential and poor prognosis.

**Key words**: oral squamous cell carcinoma, tongue, CD44v3, CD44v6, mode of invasion

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has been shown to be associated with metastasis and shorter disease-free survival (9-10). Furthermore, in HNSCC, strong CD44v3 and CD44v6 expression has been demonstrated in metastatic lymph nodes, while CD44v6 has been shown to be associated with longer disease-free survival (5).

The purpose of this study was to examine the expression patterns of CD44v3 and CD44v6 and their relationship with clinicopathological parameters in OSCC of the tongue (OSCCT).

Materials and methods

Patients

Biopsy specimens of primary OSCCT were obtained from 60 patients undergoing surgical resection at the Department of Second Oral Maxillofacial Surgery of Kyushu Dental College Hospital between 2000 and 2010. The patients included 33 males and 27 females with a mean age of 63.9 years (range 26-90 years). We used the T-category classification as described in the fourth edition of the TNM classification of the International Union Against Cancer (UICC), while we modified the N-category classification into two groups, positive or negative, for lymph node metastasis. The degree of differentiation (grade 1, well differentiated; grade 2, moderately differentiated; grade 3, poorly differentiated) and the mode of invasion, according to the Yamamoto-Kohama (YK) classification system, were assessed according to the General Rules for Clinical and Pathological Studies on Oral Cancer (11). Tissue collection was approved by the Institutional Human Ethics Committee of Kyushu Dental College (No. 09-14).

Immunohistochemical analysis

For immunohistochemical staining, 4-μm thick sections were cut from paraffin blocks. For histological examination, sections were stained with hematoxylin and eosin. After blocking endogenous peroxidase with 0.03% H2O2 in methanol for 20 min, heat-induced epitope retrieval was achieved by immersing sections in 10 mM citrate buffer (pH 6.0) and boiling for 3 min in a pressure cooker. The sections were incubated with mouse monoclonal antibodies against CD44v3 (VEF-327v3, 1:400, Novocastra Laboratories, Newcastle, UK) and CD44v6 (VEF-7, 1:400, Novocastra) overnight at 4°C. CD44v3 contains exon 8, and CD44v6 contains exon 11 inserted by alternative splicing. Sections were washed with PBS and incubated with peroxidase-conjugated anti-mouse IgG polyclonal antibody (Histofine, SimpleStain MAX-PO (MULTI), Nichirei Co., Ltd., Tokyo, Japan) for 30 min at room temperature. Peroxidase activity was detected using diaminobenzidine (ImmPACT®DAB, Vector Laboratories, CA, USA). Sections were counterstained with hematoxylin and dehydrated.

Immunoreactivity score

The percentage of positive tumor cells was quantified using Image-J software (12). CD44v3 and CD44v6 expression in tumor cells was evaluated as present or absent. Specimens with ≥25% positive cells were considered as positive (4, 6, 13-14).

Statistical analysis

Relationships between immunohistochemical expression status, lymph node metastasis, local recurrence, and secondary lymph node metastasis were analyzed using the
Mann-Whitney U-test. The Kruskal-Wallis test was used to evaluate the relationship between immunohistochemical expression status and other clinicopathological parameters (15). Survival rates were calculated by the Kaplan-Meier method and examined for statistical significance using the log-rank test. \( P < 0.05 \) was considered statistically significant.

**Results**

**Immunohistochemical profiles**

In normal epithelia (Fig. 1a), immunoreactivity for CD44v3 (Fig. 1b) and CD44v6 (Fig. 1c) was observed on the cell membranes and in the cytoplasm of basal and parabasal cells but was faint or absent in the more superficial layers. In SCC (Fig. 1d), immunoreactivity for CD44v3 (Fig. 1e) and CD44v6 (Fig. 1f) was observed in the membranes and cytoplasm of tumor cells. Reactivity for either CD44v3 or CD44v6 was not observed in the stroma and keratin pearls in the center of the tumor nests.

Significant negative correlations were present among CD44v3, the mode of invasion (Fig. 2), and the differentiation type (Fig. 3). CD44v3 expression was significantly decreased...
Table 1. Clinicopathologic parameters in relation to CD44v3 and v6 expression

<table>
<thead>
<tr>
<th>parameter</th>
<th>CD44v3</th>
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<th>CD44v6</th>
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<td></td>
<td>+†</td>
<td>−†</td>
<td>total</td>
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<tr>
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<td>5 (83.3)</td>
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<td>7 (63.6)</td>
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†+: positive, −: not-positive; *P<0.0001, **P<0.05

Clinicopathologic parameters of specimens

The relationships of clinicopathological parameters with CD44v3 and CD44v6 expression are summarized in Table 1. Decreased expression level of CD44v3 correlated with differentiation type (P<0.0001) and mode of invasion (P<0.0001). Furthermore, cases that were not-positive for CD44v3 had more frequent recurrences (P<0.05), and had secondary metastasis to lymph nodes (P<0.05). CD44v6 expression showed no correlation with clinicopathological parameters in patients with OSCCT.

CD44v3 expression and clinical outcome

Fig. 4 shows survival curves in relation to CD44v3 expression. The log-rank test of significance for Kaplan-Meier estimates of survival showed that patients with high CD44v3 expression levels had better survival rates than those with low CD44v3 expression levels (P<0.001).

Discussion

The sets of splice variants and CD44 expression levels change during the development of several malignant tumors (16). These changes can lead to altered adhesion between tumor cells and the extracellular matrix, facilitate invasion, and enhance growth (14). CD44 variant expression and its
relevance to tumor progression in various human cancers is a complicated matter. Decreased CD44 variant expression has been associated with poor outcome in melanoma and prostate and colorectal cancers (17-19). On the other hand, increased CD44 variant expression has been associated with poor survival in renal cell carcinomas and non-small-cell lung carcinomas (20-21). In OSCC, Moles et al. suggested that decreased CD44 expression increases tumor cell invasiveness and may explain the worse survival of patients with tumors. In addition, Ue et al. reported that downregulation of CD44v9 in patients with OSCC was correlated with tumor cell differentiation, and with primary and secondary metastasis to lymph nodes (8, 22).

CD44 is a transmembrane molecule with a globular N-terminal domain that helps in adhesion to extracellular matrix proteins (mainly hyaluronic acid), other glycosaminoglycans, collagen, laminin, and fibronectin (23-26). Between the globular N-terminal domain and the transmembrane domain, there is a short stalk-like region that can be enlarged by the insertion of variably spliced exon products that may contribute to ligand binding (27). It is likely that interactions with associated molecules are responsible for the complete range of functions of CD44, including the metastasis-supporting functions of CD44 variant isoforms (27). CD44v3 is modified by a heparin sulfate side chain that binds the proform of the heparin-binding growth factor (33). The binding of the scatter factor (SF) to CD44v6 is important for tumor progression. The binding of the SF to CD44v6 provides the initiating signal required for c-Met oncogene activation (28).

The TNM classification does not determine the prognosis of individual patients nor does it describe the biological characteristics of tumor cells. Therefore, it is important to seek out new, reliable prognostic factors that provide additional information regarding the biological characteristics of tumors. A histopathological classification is frequently used for prognosis in OSCC. The mode of invasion, used as a histopathological classification category, as described by Yamamoto et al., is frequently used to predict progression, metastasis, and prognosis (29-30).

In this study, we demonstrated that loss of CD44v3 expression in OSCCT was closely associated with the mode of invasion and differentiation type. Furthermore, CD44v3 expression showed a negative correlation with local recurrence, secondary metastasis to lymph nodes, and survival rate. Mark et al. reported that decreased CD44v3 expression in cutaneous melanoma correlated with poor outcome (31), which supports the results of this study. On the other hand, we found that CD44v6 expression showed no correlation with clinicopathological parameters in patients with OSCCT. The functions of these isoforms remain relatively unclear (32). However, CD44 molecules containing the v3 domain is the only CD44 isoform able to bind heparin-binding growth factors and cytokines through its heparin sulfate side chains (31, 33). For example, Grimm et al. reported that CD44v3 is modified with heparin sulfate (HS) and binds HS-binding basic fibroblast growth factor (bFGF), and they suggested that HS-binding bFGF presented by the HS-modified CD44v3 may stimulate the proliferation of normal or malignantly transformed keratinocytes in skin (34). It is hypothesized that tumor cells expressing CD44v3 may therefore accumulate matrix-bound growth factors and play a role in the control of cellular proliferation (31). This could improve the survival of tumor cells remaining in their original environment and relieve the selective pressure to progress toward a metastasis-forming phenotype (28, 31, 33).

Therefore, the loss of CD44 CD44v3 expression in tumor cells might be associated with increased metastatic potential and poor prognosis.

In conclusion, CD44v3 may be useful as a diagnostic/prognostic biomarker of OSCCT because lower CD44v3 immunoreactivity is associated with greater invasive potential, low tumor cell differentiation, and poor prognosis. On the other hand, CD44v6 expression seems to show no correlation with clinicopathological parameters in patients with OSCCT. For clinical applications, it is necessary to clarify the relationship between CD44 variant expression and the progression of OSCC.

Acknowledgments

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References


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