Role of CD44 Variant Exon 6 in Invasion of Head and Neck Squamous Cell Carcinoma

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Objectives: To determine the correlation between the expression of CD44 variant exon 6 (v6) and the clinico-pathological features of head and neck squamous cell carcinomas (HNSCCs), and to study the role of CD44v6 in cell invasion using a human HNSCC cell line (HSC-2).

Design: The expression of CD44v6 was evaluated using immunohistochemical analysis in paraffin-embedded tissue specimens from 89 primary lesions. The concentration of CD44v6 protein in 37 cryopreserved tumor specimens was evaluated using the enzyme-linked immunosorbent assay. The HSC-2 cells were treated with 2F10, a monoclonal antibody against CD44v6. The effects of 2F10 on HSC-2 cell proliferation, migration, and invasion potential were evaluated.

Results: The down-regulation of CD44v6 expression or the concentration of cancer tissue significantly correlated with a lower degree of pathohistological differentiation and a higher rate of cervical metastasis. The invasion of HSC-2 cells into type I collagen gel and the expression of CD44v6 were decreased in invading cells released from the upper layer. Furthermore, the treatment of HSC-2 cells with 2F10 significantly enhanced cell invasion. However, 2F10 did not affect either the proliferation or migration properties of HSC-2 cells.

Conclusions: The down-regulation of CD44v6 expression may be useful as a biological marker for the degree of malignancy in HNSCCs. We assume that the loss or dysfunction of CD44v6 is involved in the acquisition of invasion ability in HSC-2 cells. In addition, the potential existence of a CD44v6-mediated signal transduction pathway may play a role in inhibiting the invasion in HNSCCs.


Glycoprotein CD44 is a cell surface molecule that appears to be involved in cell-cell and cell-matrix interactions. It also appears to mediate several other functions, such as lymphocyte homing, T-cell activation, and tumor metastasis. The CD44 gene measures 50 to 60 kd, resides on chromosome 11p13, and is known to be composed of at least 20 exons. Ten of the exons are constitutively expressed on almost all cell types to produce a heavily glycosylated 85- to 90-kd isoform known as the CD44 standard form (CD44st). The remaining exons can be alternatively spliced to produce various isoforms, which are called CD44 variants (CD44v).

In 1991, the expression of some variant exons, including CD44v6, was shown to distinguish metastatic from nonmetastatic pancreatic carcinoma in the rat. Evidence that CD44 itself plays a role in metastasis was based on the fact that transfection with complementary DNA encoding those exons converted nonmetastatic rat carcinoma cells into metastatic cells. Furthermore, the injection of antibody against CD44v6-encoded peptides with metastatic cells also suppressed their metastatic behavior. Although in humans the functions of CD44 remain unclear, they are considered to play an important role in the growth and metastasis of several kinds of tumors. Attention has been drawn to the recently published report by Takahashi et al7 that CD44 plays a role in such signal transductions as cell-cell and cell-matrix interactions and thus regulates matrix metalloproteinase (MMP). They reported that cell invasion and cell migration increased and up-regulation of MMP-2 was observed in a melanoma cell line following treatment with the monoclonal antibody for CD44st.

Recent clinicopathological studies have revealed that the expression of individual variant exons was altered in several malignancies. For example, the expression of CD44v6 in gastric, colon, and breast cancers and non-Hodgkin lym-
We investigated a randomly selected sample of 89 paraffin-embedded tumor specimens that were collected from 89 patients with HNSCC in our department for 14 years (April 1, 1983, through March 31, 1997). The HNSCCs consisted of 36 oropharyngeal (OPSCCs), 18 hypopharyngeal (HPSCCs), and 35 tongue and mouth floor squamous cell (T&MFSCCs) carcinomas (Table 1). The 89 patients comprised 78 men and 11 women. The patient ages ranged from 29 to 72 years, with a mean of 57.7 years. According to the International Union Against Cancer (UICC) classification of 1997,14 11 patients had stage I; 23, stage II; 17, stage III; and 38, stage IV disease.

The immunohistochemical analyses were performed on formalin-fixed, routinely processed paraffin-embedded samples, and antigen detection was carried out by the avidin-biotin-peroxidase technique. Tissue specimens were sliced in 5-µm sections and placed on Silane-coated glass slides. Before immunohistochemical analysis was performed, the sections were deparaffinized with xylene and subjected to an antigen retrieval procedure by exposing the sections to microwaves at 600 W for 12 minutes with Soretect Target Unmasking Fluid (STUF; SEROTEC, Oxford, England). The sections were incubated with the primary monoclonal antibody 2F10 (10 µg/mL) (R&D Systems, Minneapolis, Minn), which recognizes epitopes of the CD44v6 portions, in 1% normal rabbit serum diluted with Tris-buffered saline at 4°C overnight in a humidified atmosphere. The degree of staining was assessed on a scale of 4 grades according to the percentage of cancerous cells stained: 3+, mean expression in more than 90%; 2+, 50% to 90%; 1+, 5% to 50%; and negative, less than 5% or no expression. When the epidermis adjacent to the primary cancer tissue showed strong expression of 2F10, the corresponding cancer tissue was considered to be evaluable and was subjected to this analysis (Figure 1, B). An accompanying specimen without exposure to the primary antibody was used as a negative control (Figure 1, A).

Table 1. Clinical Stage and Pathological Differentiation of Head and Neck SCCs (Immunohistochemical Analysis)*

<table>
<thead>
<tr>
<th>SCC Type</th>
<th>No. of Patients</th>
<th>Clinical Stage, No.</th>
<th>Pathological Differentiation, No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Oropharyngeal</td>
<td>36</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Hypopharyngeal</td>
<td>18</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Tongue and mouth floor</td>
<td>35</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
<td>11</td>
<td>23</td>
</tr>
</tbody>
</table>

*SCCs indicates squamous cell carcinomas.
†One patient had distant metastasis.

CD44v6 IMMUNOASSAY AND SAMPLES

We investigated a randomly selected sample of 37 cryopreserved (~80°C) tumor specimens from patients with HNSCCs who were surgically treated in our department from January 1, 1996, through December 31, 1998. The HNSCCs consisted of 3 laryngeal squamous cell carcinomas (LSCCs), 4 OPSCCs, 8 HPSCCs, and 22 T&MFSCCs (Table 2). Tumor samples were frozen until further examination to ensure that only confirmed malignant tissue was stored. Each specimen between 3 and 5 mm was thawed to room temperature, minced to a paste using a scalpel, and mixed with distilled water to make a total quantity of about 1 mL. The prepared specimen was homogenized and exposed to sonication for 30 seconds 3 times with a micro-sonication. The homogenate was centrifuged at 30000g for 1 hour. The supernatant was filtered through a 0.22-µm Millipore filter (Millipore, Bedford, Mass) before analysis.

CELL LINES

The human oral squamous cell carcinoma cell line (HSC-2)15 and the human fibrosarcoma cell line (HT-1080) were obtained from the Health Science Research Resources Bank, Osaka, Japan. Normal human fibroblasts were obtained from explants of the oral mucosa of a 2-month old girl. The HSC-2 and HT-1080 cell lines were grown in Eagle minimum essential medium (MEM) (GIBCO BRL, Grand Island, NY), and normal human fibroblasts in Dulbecco modified Eagle MEM with Ham F-12 nutrient mixture (DMEM-F-12; GIBCO) supplemented with fetal bovine serum (FBS; GIBCO), at 37°C in an atmosphere containing 5% carbon dioxide.

phoma11 was found to be associated with shorter survival. In contrast, other reports have shown the expression of CD44sv to be associated with longer survival in neuroblastoma,12 while the down-regulation of CD44v6 is associated with shorter survival in laryngeal squamous carcinoma.13 The relationship between the clinical features and the expression of CD44 isoforms in each organ remains a controversial issue. However, it is reasonable to say that the expression of the CD44 gene is specifically regulated in each organ.

In the treatment of head and neck squamous cell carcinomas (HNSCCs), the management of cervical lymphatic metastasis and primary lesions is one of the most important factors contributing to a favorable prognosis. However, cervical lymphatic metastasis cannot always be predicted from the size and extent of invasion of the pri-
CELL PROLIFERATION ANALYSIS

The effect of the monoclonal antibody 2F10 against CD44v6 on the proliferation of HSC-2 cells was assessed. The number of viable cells was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Next, 5 × 10^3 HSC-2 cells in a single-cell suspension with MEM were added to a 96-well microtiter plate (Coster Co, Cambridge, Mass). After 24 hours of incubation, the medium was completely removed and various doses of 2F10 were added. Following an additional 48 hours of incubation, the number of viable cells was measured. All experiments were performed in triplicate.

CELL MIGRATION ASSAY

The transfilter migration activity of HSC-2 cells was evaluated in a 24-well transwell (pore size, 8 µm) (Becton Dickinson, Bedford, Mass). The upper wells were placed in serum-free MEM containing 5% carbon dioxide, the cells that had not migrated to the upper surface of the membrane were removed by wiping gently with a cotton swab. Cells on the reverse side of the filter were fixed and stained with Diff-Quik (International Reagents Co, Kobe, Japan). After the inner chamber was washed in tap water, the number of migrating cells in 10 random fields was counted using light microscopy at high power (×400), and the mean ± SD was calculated. All experiments were performed in triplicate.

CELL INVASION ASSAY WITH ORGANOTYPIC RAFT CULTURE

In vitro tumor invasiveness was evaluated according to the procedures described previously, with some modifications. In a 6-well multiplate (Coster Co), 5 × 10^5 HSC-2 cells suspended in 1 mL of medium (DMEMF-12 plus 10% FBS) were seeded on gels containing 3 × 10^4 primary culture fibroblasts in 3 mL of 0.2% type I collagen neutral medium (CELLGEN; Koken Co, Tokyo, Japan). After 24 hours of incubation, the gels were detached from the wells and floated below the surface of the medium. The DMEMF-12 medium containing 10% FBS (3 mL/well) was changed every 2 days. The culture medium and gels were supplemented with various doses of 2F10. After 10 days, the composite gels were fixed with 10% neutral formalin for 15 minutes, embedded in paraffin, sectioned, and stained with hematoxylin-eosin or subjected to immunohistochemical analysis. The tumor cells that had become detached from the stratified layer and migrated into the underlying collagen gel were counted as invading cells. The number of invading cells in 5 random fields of each culture was counted under light microscopy (×200), and the mean ± SD was calculated. All experiments were performed in quadruplicate.

MMP-1 IMMUNOASSAY

For this assay, 1 × 10^5 HSC-2 cells suspended in 100 µL of medium (DMEMF-12 plus 10% FBS) in a single-cell suspension were added to a 24-well plate (Coster Co). After a 24-hour incubation, the medium was completely removed and various doses of 2F10 were added. After another 24 hours of incubation, the culture medium was filtered through a 0.22-µm Millipore filter before analysis. The conditioned medium of each well was quantified using an MMP-1 human ELISA system (Biotrak; Amersham Pharmacia Biotech Inc, Piscataway, NJ) according to the manufacturer’s instructions. Data are presented as the mean ± SD from triplicate experiments (detection limit, <1.7 ng/mL).

STATISTICAL ANALYSIS

For the statistical analysis of the expression of CD44v6, a nonparametric statistical test (Mann-Whitney test or Kruskal-Wallis test) was used. In the study of HSC-2 cell lines in vitro, data are expressed as mean ± SD. Statistical analysis was performed by means of 1-factor analysis of variance, and P values were calculated by the Fisher protected least significant difference method. P < .05 was considered statistically significant.

CORRELATION BETWEEN CD44v6 CONCENTRATION AND CLINICAL CHARACTERISTICS: ELISA

The overall mean (SD) concentration of CD44v6 in HNSCC tissues was 1.7 (1.5) µg/mg. The mean (SD) concentrations of CD44v6 were 2.2 (1.8) µg/mg in well-differentiated carcinomas and 1.22 (1.08) µg/mg in moderately and poorly differentiated carcinomas. Down-regulation of CD44v6 concentration was significantly correlated with a poorer degree of differentiation (P = .03) (Figure 2A). The mean (SD) CD44v6 concentrations were 2.1 (1.2) µg/mg in the

RESULTS

CORRELATION BETWEEN CD44v6 EXPRESSION AND CLINICAL CHARACTERISTICS: IMMUNOHISTOCHEMICAL ANALYSIS

There were many variations in the staining pattern of cancer tissues (Figure 1). For this reason, the degree of tumor staining was classified into 4 grades (see the “Materials and Methods” section). The relationships between the expression of CD44v6 and the clinicopathological factors are summarized in Table 3. In 89 cases, the positive expression rate of CD44v6 was 96% (n = 85). No correlation was found between CD44v6 expression and T stage. The rate of positive cervical lymph node metastasis increased significantly with a reduced expression of CD44v6 (P = .02). Furthermore, down-regulation of CD44v6 was significantly correlated with a poorer degree of differentiation (P = .01).
group with stage T1 and T2 tumors and 1.3 (1.8) µg/mg in the group with stage T3 and T4 tumors. With an increase in tumor size, the CD44v6 concentration in the tumor decreased significantly \((P = .02)\) (Figure 2B). The mean (SD) CD44v6 concentrations in cancer tissue were 1.05 (0.87) µg/mg for the group with cervical metastasis and 2.3 (1.7) µg/mg for the group without cervical metastasis, a significant difference \((P = .008)\) (Figure 2C).

### CD44v6 Expression in the HSC-2 Cell Line

The expression of CD44V6 was shown by immunostaining for HSC-2 cells; staining occurred chiefly in the cell membrane (Figure 3).

### Table 2. Clinical Stage and Pathological Differentiation of Head and Neck SCCs (ELISA)*

<table>
<thead>
<tr>
<th>SCC Type</th>
<th>No. of Patients</th>
<th>Clinical Stage, No.</th>
<th>Pathological Differentiation, No.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Laryngeal</td>
<td>3</td>
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<tr>
<td>Oropharyngeal</td>
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<td>1</td>
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<tr>
<td>Hypopharyngeal</td>
<td>8</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Tongue and mouth floor</td>
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<td>7</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>37</strong></td>
<td><strong>6</strong></td>
<td><strong>10</strong></td>
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*SCCs indicates squamous cell carcinomas; ELISA, enzyme-linked immunosorbent assay.

### Effect of 2F10 on HSC-2 Cell Proliferation, Migration, and Invasion

There was no difference in the cell proliferation and cell migration potential of HSC-2 cells in the presence of 2F10 at 0.1 and 1.0 µg/mL as compared with the controls (Figure 4A-B). However, in the invasion assay, the invading cell count was significantly higher in the presence of 2F10 at 0.1 µg/mL \((P = .01)\) and 1.0 µg/mL \((P = .04)\) as compared with the controls (Figure 4C, and Figure 5A-B). Moreover, in comparison with the CD44v6 immunostaining of the control specimens, CD44v6 expression of HSC-2 cells that invaded the gel after release from the cell population on the...
surface of the gel was found to be relatively low (Figure 5C).

**EFFECT OF 2F10 ON MMP-1 EXCRETION POTENTIAL OF HSC-2 CELLS**

The mean (SD) MMP-1 concentration in the culture medium was 1008.6 (50.3) ng/mL for the cells without treatment (controls) and 1100.6 (120.4) and 1189.3 (360.1) ng/mL, respectively, for the cells treated with 2F10 at 0.1 and 1.0 µg/mL. An up-regulation in MMP-1 excretion was found in response to treatment with 2F10. Moreover, in the presence of 2F10 at 1.0 µg/mL, the MMP-1 concentration increased significantly as compared with the controls ($P = .04$) (Figure 6).

**COMMENT**

Adhesion molecules play an important role in the invasion and metastasis of cancers. Since Günther et al reported in 1991 that CD44v is a factor related to the metastatic properties of cancer, the relationship between CD44v and the invasion or metastasis of cancer has been further investigated at many institutions. We confirmed an abnormal expression of CD44v in bladder, colon, and breast cancers using reverse transcription—

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**Table 3. Correlation Between CD44v6 Expression and Clinical Characteristics (Immunohistochemical Analysis)**

<table>
<thead>
<tr>
<th>Clinical Factors</th>
<th>No. of Patients (N = 89)</th>
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</tr>
<tr>
<td>Hypopharyngeal</td>
<td>18</td>
<td>1 1 3 13</td>
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<tr>
<td>Tongue and mouth floor</td>
<td>35</td>
<td>1 0 6 28</td>
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</tr>
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<td>T stage</td>
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<tr>
<td>T1-T2</td>
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<td>3 3 12 43</td>
<td>.91‡</td>
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<tr>
<td>T3-T4</td>
<td>28</td>
<td>1 2 3 22</td>
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<td>N stage</td>
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<td></td>
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<tr>
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<td>0 0 6 31</td>
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<tr>
<td>N1-N3</td>
<td>52</td>
<td>4 5 9 34</td>
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<td>Pathological differentiation</td>
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<td>Well</td>
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<tr>
<td>Moderately</td>
<td>49</td>
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</tr>
<tr>
<td>Poorly</td>
<td>13</td>
<td>4 1 1 7</td>
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</table>

*S CC indicates squamous cell carcinoma.
†Percentage of cancerous cells stained. Minus sign indicates less than 5% or no expression; 1+, more than 5% to 50%; 2+, more than 50% to 90%; and 3+, more than 90%.
‡Statistical analysis by Mann-Whitney test.
§Statistical analysis by Kruskal-Wallis test.

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**Figure 2.** Correlation between CD44 variant exon 6 (v6) concentration and degree of pathological differentiation (A), tumor stage (B), and cervical lymph node metastasis stage (C) based on the results of enzyme-linked immunosorbent assay. Data are presented as means from duplicate experiments. Error bars indicate SDs. The Mann-Whitney test was used to evaluate significance.

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**Figure 3.** Expression of CD44 variant exon 6 (v6) in head and neck squamous cell carcinoma cell line HSC-2 (CD44v6 immunohistochemical staining with Mayer hematoxylin counterstain; original magnification ×500). A, Negative control. B, CD44v6 positive expression.
polymerase chain reaction. Our results showed that the expression of CD44v differed according to organ and pathohistological type for each cancer. It has been reported3,8-11,20 that CD44v6 is one of the variants of CD44 that is closely related to metastasis and the invasion of cancers. In addition, a positive correlation has been observed between the expression of CD44v6 and the degree of malignancy of colon,9 gastric,8 uterine,20 and breast10 cancers and non-Hodgkin lymphoma.11 In contrast, in neuroblastoma 12 and laryngeal cancers, 13 decreased CD44v6 expression was found to correlate with the degree of malignancy. Therefore, when CD44v6 is regarded as an index of malignancy, organ specificity should be taken into consideration. The CD44v6 expression in HNSCCs shows characteristics different from those of cancers of other sites, which are mostly SCCs. The normal squamous mucosa of the upper airway shows strong staining for CD44v6, and down-regulation of CD44v6 is associated with cancerous changes.21

In this study using immunological staining of clinical specimens from patients with HNSCCs, the down-regulation of CD44v6 was found to correlate with a lower degree of pathohistological differentiation and a higher rate of cervical metastasis. These findings are consistent with previous reports13 on HNSCCs in which a decrease in CD44v6 expression was shown to correlate with the degree of malignancy. The expression of CD44v6 was thus shown to be a biological marker for the degree of malignancy in HNSCCs. However, the results of immunostaining revealed that CD44v6-positive cases (1+ to 3+) account for 96% of all such cases of malignancy. As a result, CD44v6 is of somewhat limited value for prediction of cer-

Figure 4. Effect of 2F10 on cell proliferation (A), cell migration (B), and cell invasion (C) in vitro by head and neck squamous cell carcinoma cell line HSC-2. Data are presented as means. Error bars indicate SDs. The Fisher protected least significant difference method was used to evaluate significance. There was no significant difference in cell proliferation or cell migration. There was a significant difference compared with control in cell invasion.

Figure 5. Effect of 2F10 on invasiveness of human head and neck squamous cell carcinoma cell line HSC-2 in organotypic raft culture without (A and C) or with (B) 2F10, 1.0 µg/mL. A and B, The invading cell count was higher in the presence of 2F10 (hematoxylin-eosin, original magnification ×80). C, The invading cells show reduction of CD44v6 expression (arrows) (immunohistochemical staining with Mayer hematoxylin counterstain; original magnification ×220).

Figure 6. Effect of 2F10 on the matrix metalloproteinase 1 (MMP-1) concentration by head and neck squamous cell carcinoma cell line HSC-2. Data are means from triplicate experiments. Error bars indicate SDs. The Fisher protected least significant difference method was used to evaluate significance.
vical metastasis. Therefore, we performed a quantitative study using the ELISA method that showed results similar to those obtained by immunological staining. A decrease in CD44v6 protein concentration was thus shown to be closely related to the acquisition of metastatic properties. Regarding the quantification of CD44v6 protein in tumor tissues by the ELISA method, measurement of the cytosol CD44v6 concentration in breast cancer cells has been reported. However, as far as we could determine, there are no previously published reports on these levels in HNSCCs. The above-described protein extraction method is an original technique developed by our group. Therefore, it is difficult to compare our findings with the results of previous reports. Nevertheless, based on these findings, we believe that the down-regulation of CD44v6 closely reflects the degree of malignancy.

Regarding the mechanism for the invasion and metastasis of tumors, we consider the most likely one to be a 3-step model consisting of the adhesion of cancerous cells to an extracellular matrix, the destruction of the matrix, and the migration of cancerous cells into the matrix. The MMPs, whose main function is the degradation of the extracellular matrix, and also some adhesive factor molecules are thought to be involved in these steps, and CD44 may be one of these factors. However, its mode of action remains to be elucidated. It was recently reported that CD44 regulates the function of signal transduction, including cell-cell and cell-matrix interactions. Moreover, Takahashi et al reported that cell invasion and cell migration were enhanced after the treatment of melanoma cells with monoclonal antibody of CD44st and the up-regulation of MMPs.

In the present study, focusing our attention on CD44v6, which showed a correlation with the cervical metastatic rate, we investigated the role played by CD44v6 in invasion and metastasis using HSC-2 cells, a CD44v6-expressing HNSCC cell line, and 2F10, a monoclonal antibody of CD44v6. For the invasion assay, type I collagen gel incorporating fibroblasts was used. This method has been used for cancer invasion models in HNSCCs for many years. Regarding adherent molecules, invasion of collagen gel has been reported to increase after treatment of esophageal cancer cell lines with the monoclonal antibody of E-cadherin. We also confirmed invasion by HSC-2 cells of collagen gel and observed that the expression of CD44v6 decreased in invading cells released from the upper layer. The addition of 2F10 to this system caused a significant increase in the number of invading HSC-2 cells. Following the addition of 2F10, the excretion of MMP-1, which belongs to the collagenase group and mainly degrades collagen type I, increased significantly in HSC-2 cells. However, the addition of 2F10 did not affect either the cell proliferation or cell migration ability of HSC-2 cells. From these results, we assumed that a loss or dysfunction of CD44v6 was thus involved in the acquisition of invasion ability in HSC-2 cells. In addition, a CD44v6-mediated signal transduction pathway may play a role in the inhibition of MMP-1 excretion. However, the level of MMP-1 was determined only by immunoassay (ELISA), and we did not determine the level of the compound in the active form. In the future, we intend to further study the mechanism for regulation of the secretion of other MMPs.

REFERENCES