ASSOCIATION OF CD44 V3-CONTAINING ISOFORMS WITH TUMOR CELL GROWTH, MIGRATION, MATRIX METALLOPROTEINASE EXPRESSION, AND LYMPH NODE METASTASIS IN HEAD AND NECK CANCER

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Abstract: Background. The CD44 family of receptors includes multiple variant isoforms, some of which have been linked to tumor progression. The objective of this study was to investigate whether CD44 v3-containing isoforms are involved in head and neck squamous cell carcinoma (HNSCC) tumor progression.

Methods. Laboratory investigation utilizing HNSCC cell lines and clinical tissue specimens was performed.

Results. Investigation of 13 HNSCC cell lines revealed a diversity of CD44 isoform profiles, including expression of CD44 v3-containing isoforms. Two cell lines, HOC313 and MDA1483, were selected for further study based on their CD44 v3 expression profile. The HOC313 cell line, which highly expresses CD44 v3-containing isoforms, demonstrated hyaluronan-mediated CD44-dependent promotion of tumor cell growth and migration. Conditioned media from the HOC313 cell line also exhibited high matrix metalloproteinase expression on gelatin zymography. Immunohistochemical analysis of a series of metastatic HNSCC lymph nodes revealed CD44 overexpression as well as staining for CD44 v3-containing isoforms.

Conclusion. CD44 v3-containing isoforms are associated with HNSCC growth, migration, and matrix metalloproteinase activity and can be identified in lymph node metastasis.

Keywords: CD44; hyaluronan; head and neck squamous cell carcinoma; tumor progression; metastasis

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide.¹ Advanced-stage HNSCC continues to have poor 5-year survival rates (0% to 40%) which have not significantly improved in the last 30 years. Understanding the mechanisms underlying HNSCC tumor growth, invasion, and metastasis are critical to improving outcome for this disease.

Hyaluronan (HA), a glycosaminoglycan component of the extracellular matrix (ECM), is the primary ligand for the CD44 membrane receptor. Recently, HA has been studied with regard to its interaction with various CD44-mediated signaling pathways.²³ HA-CD44 signaling has been linked to tumor progression, including invasion and metastasis, as well as chemotherapy resistance in several tumor models.⁴⁻⁸ We previously

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reported that HA-CD44 promotes epidermal growth factor receptor (EGFR)-mediated oncogenic signaling and chemotherapy resistance in HNSCC cell lines.9,10

The CD44 gene contains 19 exons. Up to 10 exons (primarily exons 6–14) may be alternatively spliced to give rise to multiple variant CD44 (CD44v) isoforms, which along with CD44s (approximately 85-kDa isoform with no variable exons) make up the CD44 class of receptors (Figure 1).3,11 The multiple splicing possibilities of the variable exons of CD44 could theoretically give rise to a vast number of CD44 variants, although relatively few have been described. Various CD44v isoforms are differentially expressed in many normal and malignant cell types, and confirmation of CD44 isoform expression in HNSCC, both in tissue specimens and established cell lines, is well documented.4–6,9,11–13 HA-CD44 interaction and signaling may involve CD44s or other CD44v isoforms.

Overexpression of several CD44 variants has been associated with tumor progression.4–6,11,12 We previously described a novel CD44 v3 isoform involved in HNSCC progression.6 However, other studies have reported a correlation between increased tumor progression and decreased expression of certain CD44 variants.12,13 Thus, further studies to elucidate the relationship between CD44 variant isoform expression and HNSCC progression are needed.

Treatment of patients with HNSCC is limited by variable response to standard therapy. Identification of the molecular factors that correlate with advanced disease, outcome, and response to treatment will enable clinicians to choose the best treatment for patients, as well as provide new targets for the development of novel therapies. In the current investigation, our hypothesis was that CD44 v3-containing isoforms are involved in HA-dependent tumor progression behaviors and would be identified in metastatic HNSCC. We screened 13 HNSCC cell lines for CD44 isoform expression in order to identify expression of CD44 v3-containing isoforms. Two cell lines, selected on the basis of their CD44 v3-containing isoform expression status, were further studied for growth, migration, and matrix metalloproteinase (MMP) expression. Finally, a series of metastatic HNSCC lymph nodes were examined for CD44 and CD44 v3 protein expression.

**MATERIALS AND METHODS**

**Clinical Tumor Samples and Cell Cultures.** Institutional Review Board approval was obtained from the University of California, San Francisco (UCSF) Committee on Human Research for all tissue collection. The clinical tissue specimens were portions of cervical lymph nodes and normal mucosa (distant from primary tumor) of patients undergoing surgical treatment of squamous cell carcinomas from multiple primary sites of the upper aerodigestive tract at the San Francisco VA Medical Center. Thirteen primary HNSCC cell lines (MDA686, MDA1386, MDA886, SCC15, 1483, 584, MSK921, HOC313, SCC31, SCC10A, MDA1586, MDA1186, HSC3, SCC4) and 1 oral keratinocyte primary cell culture were used in our experiments. HOC313 was selected for additional studies because immunoblotting and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) data revealed high expression of a CD44 v3-containing isoform. 1483, which had the lowest total CD44 expression among our panel of cell lines, was selected as a control. The HNSCC cell lines were gifts from Dr. Bhuvesh Singh of the Memorial Sloan-Kettering Cancer Center, New York, NY, and Dr. Randall Kramer of the UCSF School of Dentistry. The oral keratinocyte primary cell culture was obtained from the San Francisco VA Medical Center Core Tissue Culture facility. HNSCC cells were maintained in Dulbecco’s modified Eagle’s medium or Minimal Essential medium supplemented with...
10% fetal bovine serum. Cells were routinely serum-starved (and therefore deprived of serum HA) before adding HA.

**Antibodies and Reagents.** Monoclonal rat anti-human CD44 antibody (clone: 020; isotype: IgG2b), which recognizes a common determinant of the CD44 class of glycoproteins, was obtained from CMB-TECH, Inc. (San Francisco, CA). Polyclonal rabbit anti-human CD44v3 antibody (lot no. D21548: IgG), which was produced using a synthetic peptide of the human CD44 variant isoform containing the v3 exon (exon 7), was obtained from CalBiochem (EMD Biosciences, San Diego, CA). High molecular mass HA polymers (approximately 500,000-Da polymers) obtained from Healon (Pharmacia, Erlangen, Germany) were prepared by gel filtration chromatography using a Sephacryl S1000 column. The purity of the high molecular mass HA polymers used in our experiments was further verified by anion exchange high performance liquid chromatography.

**Real-Time Reverse Transcriptase-Polymerase Chain Reaction.** Total RNA from cells was extracted with TRIzol reagent (Life Technologies, Inc., Grand Island, NY) following the manufacturer’s protocol and repurified by the RNeasy Mini-spin protocol (Qiagen, Valencia, CA). RNA samples were treated before analysis with DNAse I to eliminate residual genomic DNA. Two micrograms of total RNA were reversed transcribed with MultiScribe Reverse Transcriptase (Applied Biosystems, Inc., Foster City, CA). Primers to amplify all isoforms of CD44 and to amplify the CD44 expression were calculated using the 2-Ct method. To adjust for variations in loading, the Ct values for each gene were normalized against the Ct values for the housekeeping gene, glyceraldehyde-3-phosphatedehydrogenase. Relative gene expression was calculated using the 2-Ct method.

**Immunoblotting Techniques.** After growing in serum-free media for 24 hours, cells lysates were obtained, separated by gel electrophoresis, and blotted onto nitrocellulose filters as described previously.9,10 The nitrocellulose filters were incubated with rat anti-CD44 antibody, or rabbit anti-CD44v3 antibody, followed by incubation with horseradish peroxidase-labeled anti-rat or anti-rabbit IgG. The blots were then developed by the enhanced chemiluminescence system (ECLTM system, Amersham Life Sciences, Piscataway, NJ).

**MTT Growth Assays.** Logarithmically growing cell lines were cultured, washed, counted, and plated at 3000 cells per well in 96-well plates and incubated in serum-free media overnight. The following day, the cells were treated with or without HA (50 µg/mL) and/or anti-CD44 antibody, or anti-CD44v3 antibody. Zero, 24, 48, and 72 hours later, MTT assays were performed according to the manufacturer’s protocol (Roche Diagnostics, Mannheim, Germany), as described previously.9,10 Cell growth for each experiment is expressed relative to the growth of untreated HOC313 cells at 24 hours, which has been assigned an arbitrary value of 1.0. Each triplicate assay was repeated at least 3 times.

**Tumor Cell Migration Assays.** Twenty-four Transwell units were used for monitoring in vitro cell migration as described previously.5,14 Specifically, 8-µM porosity polycarbonate filters (Costar Corp., Cambridge, MA) were used for the cell migration assay. Cells in the presence or absence of anti-CD44 antibody (50 µg/mL) or anti-CD44v3 antibody (50 µg/mL) were placed in the upper chamber of the Transwell unit. Medium with or without HA (50 µg/mL) was placed in the lower chamber of the Transwell unit. After 18-hour incubation at 37°C, cells on the upper side of the filter were removed by wiping with a cotton swab. Cell migration processes were determined by measuring the cells that migrated to the lower side of the polycarbonate filters. Each assay was performed in triplicate and repeated at least 3 times. The number of tumor cells that migrated in untreated HOC313 cells (control) was arbitrarily designated 100%.

**Gelatin Zymography.** Gelatin zymography was used for detecting MMPs. Serum-free conditioned
media was obtained from HOC313 and 1483 cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 7.5% polyacrylamide containing 0.33 mg/mL gelatin. The gels were incubated in assay buffer at 37°C for 18 hours. Gels were then stained with Coomassie blue R250. Both latent and active forms of gelatinases or MMPs produce clear areas in the gel.

**Immunohistochemistry.** The tissue specimens were fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 24 hours at 4°C and embedded in paraffin. Five micrometer–thick tissue sections were placed on positively charged glass slides (Fisher Scientific, Pittsburgh, PA). Immunohistochemical stains were performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA), according to the manufacturer’s protocol. Anti-CD44 antibody or anti-CD44v3 antibody was applied to tissue sections and incubated overnight at 4°C. Secondary biotinylated antibody and streptavidin-HRP conjugate complex were applied for 60 and 30 minutes, respectively. After washing in buffer, the chromogen dianaminobenzidine was applied for 5 minutes followed by a counterstain with Mayer’s hematoxylin. Negative controls included substituting the primary antisera with preimmune sera from the same species and omitting the primary antibody.

**RESULTS**

**CD44 and CD44 v3-Containing Isoform Expression in Primary Oral Keratinocytes and HNSCC Cell Lines.** Total RNA was extracted from primary oral keratinocytes and 13 HNSCC cell lines. Real-time RT-PCR reactions were performed using primers which amplify all isoforms of CD44 (Table 1). The CD44 RNA expression for each HNSCC cell line is expressed as a relative ratio to the CD44 RNA expression of the OKC cells. As Table 1 indicates, several HNSCC cell lines demonstrated moderate overexpression and underexpression of CD44 v3, and 1 cell line, HOC313, demonstrated a 3-fold overexpression of CD44 v3 RNA compared with OKC. Although using the OKC cells as a single reference cannot allow determination of true overexpression or underexpression, data from Table 1 do allow relative comparisons of CD44 expression among the HNSCC cell lines. Next, lysates from the OKC cell cultures and the same HNSCC cell lines were probed with an antibody specific for CD44 v3-containing isoforms. Several of the HNSCC cell lines demonstrated slight expression of CD44 v3-containing isoforms (data not shown), and the lysate from 1 cell line, HOC313, resulted in an immunoblot demonstrating a strong band at 115 kDa (Figure 2B). Taken together, the RT-PCR and immunoblotting results from our HNSCC cell lines reveal a diversity of CD44 isoform expression, including 1 cell line, HOC313, which highly expresses a CD44 v3-containing isoform. This cell line was selected for further functional phenotype analysis. 1483, which did not express CD44 v3-containing isoforms and expressed the least total CD44 RNA or protein among our panel, was selected as a control for subsequent experiments.

**CD44 v3-Containing Isoform Expression is Associated with Increased Tumor Cell Growth.** To test the hypothesis that CD44 v3-containing isoform expression would correlate with tumor progression behaviors, in vitro growth assays were performed on the HOC313 and 1483 cells with or

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**Table 1. CD44 and CD44 v3 RNA expression in 13 head and neck squamous cell carcinoma cell lines.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ratio of CD44 RNA expression relative to oral keratinocytes</th>
<th>Ratio of CD44 v3 exon RNA expression relative to oral keratinocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA686</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>MDA886</td>
<td>1.8</td>
<td>1.2</td>
</tr>
<tr>
<td>MDA1386</td>
<td>2.5</td>
<td>1.3</td>
</tr>
<tr>
<td>MDA1586</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>SCC15</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>1483*</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>584</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>MSK921</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>HOC313*</td>
<td>1.1</td>
<td>3.1</td>
</tr>
<tr>
<td>SCC10A</td>
<td>1.2</td>
<td>1.5</td>
</tr>
<tr>
<td>MDA1186</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>HSC3</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td>SCC4</td>
<td>1.6</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*Cell lines selected for further study.*
without HA treatment (50 μg/mL), or pretreatment with anti-CD44 antibody (blocks ability of HA to bind to CD44 receptors) followed with or without HA treatment, or anti-CD44v3 antibody (binds to CD44 v3 glycoaminoglycan attachment sites) treatment (Figure 3). Cell proliferation was measured at 0, 24, 48, and 72 hours. Compared with HOC313, the low-CD44 expressing cell line 1483 demonstrated less growth, and its proliferation was not significantly altered by any of the treatments used in the experiment (data not shown). On the other hand, the HOC313 cell line demonstrated increased growth with HA treatment that was eliminated by pretreatment with an antibody which blocks HA-binding to the CD44 receptor. Treatment with CD44 antibody alone did not affect cell growth, but treatment with anti-CD44v3 antibody, which binds to the active glycoaminoglycan attachment sites of the v3 exon, resulted in inhibition of HOC313 cell growth. These results suggest that CD44 v3-containing isoform expression is associated with promotion of HA-dependent tumor cell growth.

**CD44 v3-Containing Isoform Expression is Associated with Increased Tumor Cell Migration.** Tumor cell migration assays were performed on the HOC313 and 1483 cells with or without HA treatment (50 μg/mL), or pretreatment with anti-CD44 antibody followed with or without HA treatment.

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**FIGURE 2.** CD44 and CD44 v3 protein expression in head and neck squamous cell carcinoma (HNSCC) cell lines. (A) Immunoblot using anti-CD44 antibody (panel a) and anti-β-actin antibody (panel b) of cell lysates from 13 HNSCC cell lines (lane 1: MDA686; 2: MDA886; 3: MDA1386; 4: SCC15; 5: 1483*; 6: 584; 7: MSK921; 8: HOC313*; 9: SCC10A; 10: MDA1586; 11: MDA1186; 12: HSC3; 13: SCC4) and 1 oral keratinocyte primary cell culture (lane 14: OKC). (B) Immunoblot using anti-CD44v3 antibody of lysates from HOC313* (lane 1), 1483* (lane 2), and OKC (lane 3). *Two cell lines selected for further study. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
or anti-CD44v3 antibody treatment (Table 2). Compared with HOC313, the low-CD44 expressing cell line 1483 demonstrated less cell migration, and its migration was not significantly altered by any of the treatments used in the experiment. On the other hand, the HOC313 cell line demonstrated increased migration with HA treatment that was eliminated by pretreatment with an antibody which blocks HA-binding to the CD44 receptor. Treatment with anti-CD44 antibody alone did not affect cell migration, but treatment with anti-CD44v3 antibody, which binds to the active glycoaminoglycan attachment sites of the v3 exon, resulted in inhibition of HOC313 cell migration. These results suggest that CD44 v3-containing isoform expression is associated with promotion of HA-dependent tumor cell migration.

**HOC313 Cells Highly Express Both CD44 v3-Containing Isoforms and Matrix Metalloproteinases.** Metastatic tumor cells are capable of degrading the ECM barrier to migrate out of the primary tumor location and establish new sites of metastasis. The breakdown of the ECM can be traced to the action of one or more MMPs.\textsuperscript{15,16} MMPs are secreted as proenzymes and become activated outside the cell. Several MMPs, including the 72-kDa gelatinase, MMP-2, and the 92-kDa gelatinase, MMP-9, are likely responsible for ECM degradation as a prelude to invasion and metastasis. MMP expression has previously been linked to CD44 v3-containing isoform expression and HA-mediated invasion in breast carcinoma.\textsuperscript{15,16} To investigate a putative role for CD44 v3 isoforms in HNSCC invasion and metastasis, serum-free conditioned media from the HOC313 and 1483 cells were obtained, and gelatin zymography performed (Figure 4). The conditioned media from HOC313

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**Table 2.** In vitro tumor cell migration assays.

<table>
<thead>
<tr>
<th>Tumor cell migration (%)</th>
<th>HOC313</th>
<th>1483</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>33</td>
</tr>
<tr>
<td>+HA</td>
<td>333</td>
<td>36</td>
</tr>
<tr>
<td>+HA +CD44 antibody</td>
<td>107</td>
<td>32</td>
</tr>
<tr>
<td>+CD44 antibody</td>
<td>105</td>
<td>35</td>
</tr>
<tr>
<td>+CD44v3 antibody</td>
<td>67</td>
<td>31</td>
</tr>
</tbody>
</table>

Cell migration assays with and without HA (50 µg/mL), or pretreated with anti-CD44 antibody followed with or without HA, or treated with anti-CD44v3 antibody, were performed with HOC313 and 1483 cells. Table displays relative migration processes of the cell lines with various treatments. Migration of control HOC313 cells has been arbitrarily designated as 100%. Each value represents the mean of at least 3 experiments. HA: hyaluronan.

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**FIGURE 3.** In vitro tumor cell growth assays. HOC313 or 1483 cells were grown with and without hyaluronan (HA) (50 µg/mL), or pretreated with anti-CD44 antibody followed with or without HA, or treated with anti-CD44v3 antibody. MTT growth assays were performed at 0, 24, 48, and 72 hours. Graph displays relative growth of the cell lines. Growth of 1483 cells was not significantly altered with any of the treatments used (data not shown). Cell growth of HOC313 without HA at 24 hours has been arbitrarily designated as 1.0; error bars were determined from standard deviations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

**FIGURE 4.** In vitro tumor cell matrix metalloproteinase (MMP) expression. Conditioned media from cells grown in serum-free culture were obtained from HOC313 (lane 2) and 1483 cells (lane 3), and gelatin zymography performed as described in “Materials and Methods.” Lane 1 is conditioned media containing MMP-2 and MMP-9.
revealed strong expression of both MMP-2 and MMP-9 as well as evidence of other smaller MMPs; on the other hand, MMP expression of 1483 was less. Although the 2 cell lines likely differ in expression of other factors besides CD44, the zymography results are consistent with previous studies linking CD44 v3 isoform and MMP expression, with consequent greater capability for invasion and metastasis.

**CD44 v3-Containing Isoform Expression in Head and Neck Squamous Cell Carcinoma Lymph Nodes.** To confirm the clinical relevance for CD44 v3 isoform expression in HNSCC, 9 metastatic neck lymph nodes, and matched normal mucosa, were examined by immunohistochemistry using an antibody against a common determinant of all CD44 isoforms and a specific antibody for CD44 v3-containing isoforms. The normal mucosa revealed CD44 staining, but no CD44 v3 staining. Seven of 9 nodes showed intense membrane staining of CD44, and 6 nodes also exhibited staining for CD44 v3, suggesting that CD44 and CD44 v3-containing isoforms may be differentially expressed in HNSCC metastasis (Figure 5).

**DISCUSSION**

CD44 comprises a family of isoforms that are important cell membrane receptors found in a variety of normal and malignant cells. The various CD44 isoforms result from the alternative splicing of up to 10 variable exons. CD44 receptors are situated at the interface of the cytoplasm and ECM, and all CD44 isoforms consist of a conserved extracellular HA-specific binding region and a conserved intracellular ankyrin binding region. The CD44 receptor linkage between the external environment and cytoskeleton allows one family of molecules to mediate a variety of diverse cellular behaviors including mitogenesis, adhesion, and migration. CD44 variant isoforms are differentially expressed in some tumors. CD44 variant isoforms, with their conserved extracellular and cytoplasmic regions but altered protein core, have been shown to be capable of significantly altering cell behavior. Studies with HNSCC and other tumors have demonstrated that various CD44 variant isoforms promote tumor progression behaviors including growth, invasion, and metastasis.

Evidence is accumulating regarding the role of CD44 v3-containing isoforms in tumor progression. Several studies have described the unique biologic characteristics of the v3 exon, which contains glycoaminoglycan attachment sites and has been shown to be capable of binding various growth factors. Thus, the binding capabilities of the v3 exon may allow CD44 v3-containing isoforms to function as growth factor receptors, transmitting oncogenic signals to promote tumor proliferation, migration, and metastasis. The identity of the molecular factors that bind and transmit oncogenic signaling mediated by CD44 v3-containing isoforms are yet to be fully described. However, supporting the validity of this mechanism is the observation that CD44 v3-containing isoforms have been found to be associated with breast carcinoma metastasis, poor prognosis in colon carcinoma, and the visceral metastatic phenotype of malignant melanoma. CD44 v3-containing isoforms were also shown to promote invasive behavior in colon carcinoma and in synoviocytes of patients with rheumatoid arthritis. We reported that a novel CD44 v3-containing isoform is involved in HNSCC pro-
CD44 v3 Isoforms in Head and Neck Cancer

REFERENCES