Clinicopathological Significance of Expression of CD44 Variants in Head and Neck Squamous Cell Carcinoma

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Splice variants of the cell surface glycoprotein CD44 have been reported to be associated with the progression of various human tumors. The aim of this study is to determine the correlation between the expression of CD44 isoforms, especially CD44 variant 2 (CD44v2), and the clinicopathological features of head and neck squamous cell carcinomas (HNSCCs). The expression of CD44 isoforms was evaluated immunohistochemically in paraffin-embedded tissues from 89 primary lesions, using monoclonal antibodies against CD44 standard (CD44st), CD44 variant 6 (CD44v6) and CD44v2. Cancer tissues from 89 (100%), 85 (95.5%) and 59 (66.3%) patients showed positive immunoreactivity for CD44st, CD44v6 and CD44v2, respectively. A significant correlation was observed between the down-regulation of CD44v2 and poorer differentiation of the tumor cells (P=0.02). We could not find any significant correlation between the expression of CD44v2 and T stage or N stage (lymph node status). However, the rate of positive cervical lymph node metastasis tended to increase with reduced expression of CD44v2 (P=0.08). Down-regulation of CD44v2 expression was correlated with shorter overall survival (P=0.01). Furthermore, Cox's multivariate analysis revealed that only CD44v2 expression and lymph node status were independent prognostic factors. These findings suggest that down-regulation of CD44v2 expression may be one of the biological markers for the degree of malignancy in HNSCCs.

Key words: CD44 variant — Head and neck squamous cell carcinoma — Immunohistochemistry — Prognostic factor — Biological marker

Glycoprotein CD44 is a cell surface molecule which appears to be involved in cell-cell and cell-matrix interactions.1, 2) It also appears to mediate several other functions, such as lymphocyte homing,3) T cell activation4) and tumor metastasis.4, 5) The CD44 gene is 50–60 kD in size, resides on chromosome 11p13, and is known to be composed of at least 20 exons. Ten of them are constitutively expressed on almost all cell types to produce a heavily glycosylated 85–90 kD isoform known as the standard form (CD44st). The remaining exons can be alternatively spliced to produce various isoforms,5–7) which are called CD44 variants (CD44v). Although in humans the functions of CD44v remain unclear, they may play an important role in the growth and metastasis of several kinds of tumors.2, 8, 9)

Recent clinicopathological studies have revealed that the expression of individual variant exons is altered in several malignancies. For example, the expression of CD44v9 in gastric cancer,10) CD44v6 in colon cancer,11) CD44v8–v10 in colon cancer,12) CD44v6 in breast cancer13) and CD44v6 in non-Hodgkin lymphoma14) was associated with shorter survival. Additionally, using a recently developed monoclonal antibody against a CD44v2 epitope, we have demonstrated that CD44v2 expression was correlated significantly with poorer prognosis in breast cancer15) and in pancreatic cancer.16) Other reports show that the expression of CD44st is associated with longer survival in neuroblastoma17) and that down-regulation of CD44v6 is associated with shorter survival in laryngeal squamous cell carcinoma.18) We have proved that down-regulation of CD44v2 was correlated significantly with poorer prognosis in esophageal squamous cell carcinoma.19) The precise relationship between the clinical features and the expression of CD44 isoforms in each organ is a controversial issue. The data, however, indicate that the expression of the CD44 gene is under the specific regulation of each organ. With this background, we studied the correlation between the expression of the CD44 gene and various clinicopathological indices in head and neck squamous cell carcinomas (HNSCCs). Surgical specimens or biopsy samples from primary HNSCCs were analyzed immunohistochemically using commercially available monoclonal antibodies against CD44st and CD44v6 and a newly developed antibody against CD44v2.
MATERIALS AND METHODS

Patients and samples Eighty-nine HNSCC lesions were examined in this study. Samples were collected from 89 HNSCC patients in our department during the 14 years from 1983 through 1997. The HNSCCs consisted of 36 oropharyngeal (OPSCCs), 18 hypopharyngeal (HPSCCs), and 35 tongue and mouth floor squamous cell carcinomas (T&MFSCCs)(Table I). The 89 patients comprised 78 males and 11 females. The age of the patients ranged from 29 to 72 years, with a mean of 57.7 years. The follow-up observation span was 190 months at the longest and 8 months at the shortest, with a mean observation period of 63.5 months. Eleven patients had stage I, 23 stage II, 17 stage III, and 38 stage IV cancers according to the UICC classification of 1987. Among the 89 patients, curative treatment was attempted for 84 patients. We analyzed the overall survival in these 84 patients. Five patients who received only palliative treatment were excluded from the survival analysis. Histological preparations from biopsy or operation in these patients were used as the materials for this study. A total of 89 histological specimens from the primary lesions were examined. All samples were fixed in 10% formalin and embedded in paraffin.

Immunohistochemistry Tissues were sliced in 5-µm sections, and placed on silane-coated glass slides. Before immunohistochemistry, the sections were deparaffined with xylene and subjected to an antigen retrieval procedure by microwaving at 600 W for 12 min with STUF (Dainippon Pharmaceutics, Nagoya). Nonspecific binding of primary antibodies was blocked by incubation with 20% normal rabbit serum at 37°C for 60 min. Next, the sections were incubated with the primary monoclonal antibody 2c5 (3.3 µg/ml, R&D Systems, Abingdon, UK), 2F10 (10 µg/ml, R&D Systems) or M23.6.1 (20 µg/ml),20) recognizing epitopes of the CD44st, CD44v6 and CD44v2 portions, respectively, in 1% normal rabbit serum diluted with Tris-buffer saline (TBS) at 4°C overnight in a wet box. Afterwards the sections were treated with 0.3% H2O2 in 100% methanol for 10 min to inactivate the endogenous peroxidase. The sections were incubated with a 1:400 dilution of biotinylated anti-mouse IgG (DAKO, Santa Barbara, CA) at room temperature for 60 min, and then with horseradish peroxidase-conjugated avidin-biotin complex (DAKO) at room temperature for 60 min. Immunostaining was visualized with 3,3-diaminobenzidine (Sigma, St. Louis, MO) for 20 min, and the reaction was stopped by washing with water. Sections were counterstained with Mayer’s hematoxylin, air-dried, and mounted. Negative controls included sections treated with 1% normal rabbit serum alone in place of the primary antibody. The degree of staining was assessed on a 5-point scale (0=negative, 4=strong expression). Staining was defined as positive if more than 5% of tumor cells had a score of 2 or more. When the epidermis adjacent to the primary cancer tissue strongly expressed any antibody, the corresponding cancer tissue was considered to be evaluable and was subjected to this study (Fig. 1, B, C and D). An accompanying control, without primary antibody, was used as a negative control (Fig. 1A).

Statistical analysis For the statistical analysis of the expression of CD44 isoforms, Fisher’s exact test was used. For the analysis of overall survival rate, Kaplan-Meier’s method21) was used together with the log-rank test. To examine the relation between combinations of markers and survival times, a Cox’s proportional hazards regression model22) was used.

RESULTS

Normal squamous mucosa, but not keratinized surface epithelium, showed strong staining with any monoclonal antibody in all specimens. However, in the case of CD44v2 expression, the basal layer of the squamous membrane was very weakly stained. In stromal cells, CD44st was expressed, but CD44v2 and CD44v6 were not expressed in any specimen of non-cancer tissues (Fig. 1). There were many variations in the staining pattern of cancer tissues (Fig. 2).

The clinical stage and pathological differentiation are summarized for every primary lesion of HNSCC (Table I). In the 89 cases, the positive expression rates of CD44st,
CD44v6 and CD44v2 were 100%, 95.5% and 66.3%, respectively. The negative group for CD44v6 consisted of only 4 patients. They were all also negative for CD44v2.

The relation between the expression of CD44v2 and the clinicopathological factors is summarized in Table II. The expression of CD44v2 was not correlated significantly

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**Fig. 1.** Expression of CD44 isoforms in normal mucosa. (A) Negative control. (B) Expression of CD44st: basal lamina and fibrous connective tissue (except for stratum corneum) were stained. (C) Expression of CD44v6. (D) Expression of CD44v2: staining pattern was similar to that of CD44v6, though basal layers were faintly stained (arrowhead). Scale bar 100 μm.

**Fig. 2.** Expression of CD44 variants in HNSCCs. (A) CD44v6 strong expression in tongue carcinoma (well differentiated). (B) CD44v2 mild expression in oropharyngeal carcinoma (moderate differentiated). Scale bar 50 μm.
Table II. Correlation between CD44v2 Expression and Clinical Characteristics

| Clinical factors          | No. of patients | CD44v2 expression | \( P \) value |
|---------------------------|-----------------|-------------------|---------------|
| Primary lesion            |                 |                   |               |
| T&MFSCCs                  | 35              | 9                 | 26            | 0.14          |
| others                    | 54              | 21                | 33            |               |
| T classification          |                 |                   |               |
| \( T_{1-2} \)             | 61              | 22                | 39            | 0.32          |
| \( T_{3-4} \)             | 28              | 8                 | 20            |               |
| N classification          |                 |                   |               |
| \( N_0 \)                 | 37              | 9                 | 28            | 0.08          |
| \( N_{1-3} \)             | 52              | 21                | 31            |               |
| Differentiation\(^a\)     |                 |                   |               |
| well, moderate            | 76              | 22                | 54            | 0.02          |
| poorly                    | 13              | 8                 | 5             |               |

\(^a\) Pathological differentiation.
T&MFSCCs, tongue and mouth floor squamous cell carcinomas; others, oropharyngeal and hypopharyngeal squamous cell carcinomas.

Table III. Results of Univariate Analysis and Cox’s Multivariate Analysis in Head and Neck Squamous Cell Carcinomas

| Risk factors          | Overall survival (%)\(^a\) | Univariate \( P \) value\(^b\) | Multivariate \( P \) value | Relative risk | 95\%CI\(^c\) |
|-----------------------|-----------------------------|-------------------------------|---------------------------|----------------|---------------|
| Gender                |                             |                               |                           |                |               |
| male                  | 66.7                        | 0.36                          | 0.38                      | 0.52           | 0.11–2.28     |
| female                | 75.0                        |                               |                           |                |               |
| Age                   |                             |                               |                           |                |               |
| \( 60 \leq \)         | 67.8                        | 0.91                          | 0.49                      | 1.38           | 0.54–3.47     |
| \( 59 \leq \)         | 68.2                        |                               |                           |                |               |
| Primary lesion        |                             |                               |                           |                |               |
| T&MFSCCs              | 73.5                        | 0.62                          | 0.47                      | 1.40           | 0.55–3.57     |
| others                | 63.9                        |                               |                           |                |               |
| T classification      |                             |                               |                           |                |               |
| \( T_{1-2} \)         | 72.9                        | 0.09                          | 0.16                      | 0.51           | 0.20–1.30     |
| \( T_{3-4} \)         | 57.3                        |                               |                           |                |               |
| N classification      |                             |                               |                           |                |               |
| \( N_0 \)             | 89.2                        | 0.007                         | 0.03                      | 3.41           | 1.08–10.72    |
| \( N_{1-3} \)         | 55.9                        |                               |                           |                |               |
| Differentiation\(^d\) |                             |                               |                           |                |               |
| well, moderate        | 68.6                        | 0.77                          | 0.98                      | 1.02           | 0.21–4.85     |
| poorly                | 59.7                        |                               |                           |                |               |
| CD44v2 expression     |                             |                               |                           |                |               |
| positive              | 75.9                        | 0.01                          | 0.01                      | 0.32           | 0.13–0.79     |
| negative              | 51.6                        |                               |                           |                |               |

\(^a\) Kaplan-Meier analysis of overall survival.
\(^b\) Statistical analysis by log-rank test.
\(^c\) Confidence interval.
\(^d\) Pathological differentiation.
T&MFSCCs, tongue and mouth floor squamous cell carcinomas; others, oropharyngeal and hypopharyngeal squamous cell carcinomas.
with the site of primary lesion, T stage or N stage (lymph node status). Cases demonstrating positive cervical lymph node metastasis tended to show a reduced expression of CD44v2 ($P = 0.08$). CD44v2 expression was down-regulated in cancers with a poorer degree of differentiation ($P = 0.02$). Fig. 3 demonstrates the relationship between expression of CD44v2 and overall survival. Patients with CD44v2-positivity had significantly longer survival than those with CD44v2-negativity ($P = 0.01$). Furthermore, in the Cox’s multivariate analysis using factors including sex, age, site of primary lesion, T stage, N stage and pathological differentiation, only the N stage ($P = 0.03$) and the expression of CD44v2 ($P = 0.01$) independently influenced the survival rate (Table III).

**DISCUSSION**

Since Günthert et al. reported that a particular form of CD44v may be directly involved in the metastasis of cancer, the expression patterns of the CD44 isoforms in various cancers have been assessed by numerous investigators. Up to now, a positive expression of CD44v has been associated with both tumor metastasis and a poorer prognosis in non-Hodgkin lymphoma, colon cancer and breast cancer. However, the absence of CD44st expression was found to be significantly correlated with a lower survival rate in neuroblastoma. Moreover, the down-regulation of CD44v6 has also been reported to be related with malignant changes in the epithelium in HNSCCs. In addition, other reports have described a correlation between a down-regulated expression of CD44v6 and CD44st and regional lymph node metastasis in HNSCCs. Based on these findings, it appears that the expression pattern of CD44v varies according to the involved organ, and therefore some organ specificity appears to exist regarding CD44v expression. However, the mechanisms of specific expression of CD44v in various human cancers remain unclear.

We find that CD44v6 was expressed in 95.5% of HNSCCs, a result similar to those obtained in the previous studies. We did not find the expression of CD44v6 to be a discerning biological marker. The survival rate in the negative group for CD44v2 was, however significantly lower than that in the positive group at all stages. In addition, the expression of CD44v2 is an independent prognostic factor in multivariate analysis. Based on these findings, the expression of CD44v2 is considered to be an important prognostic factor in HNSCCs.

Fujita et al. reported that the down-regulation of CD44 in endometrial cancer cells may cause a reduction in the adhesiveness between cells, as well as between the cell and the basement membrane. They suggested that the down-regulation of CD44 resulted in easy detachment of cancer cells from the rigid cancer nest and therefore such down-regulation might be an indicator of high metastatic potential. Moreover, it was recently reported that CD44 regulates signal transduction, including cell-cell and cell-matrix interactions. Takahashi et al. reported that cell invasion and cell migration were enhanced after the treatment of melanoma cells with a monoclonal antibody to CD44 and the up-regulation of matrix metalloproteinases. We therefore consider that loss or dysfunction of CD44 may play an important role in cancer invasion or metastasis. Our present findings show that loss or dysfunction of CD44v2 is involved in cancer spread, and predicts a poor prognosis of patients with HNSCCs.

**ACKNOWLEDGMENTS**

This work was supported in part by grants from Keio University and the Ministry of Education, Science, Sports and Culture of Japan (Grant No. 10770906). We thank Miss K. Kobayashi, Department of Medical Oncology, National Cancer Center Hospital for technical support. We also thank Professor Y. Inuyama, Department of Otolaryngology, Hokkaido University School of Medicine for his careful review of this manuscript and for helpful advice.

(Received November 10, 1999/Revised January 31, 2000/Accepted February 8, 2000)
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