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# Expression of CD44 Variant Exons 8–10 in Gastric Cancer

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**The expression of CD44 variant containing variant exons 8–10 product (CD44v8–10) was studied by western blot analysis and immunohistochemistry in gastric cancers using a monoclonal antibody, 44-1V. On western blots, a single band of 130 kD was recognized in stomach cancer cell lines. CD44v8–10 expression, with reactivity localized in the cell membrane, was found in 65 (33.5%) of the 194 advanced gastric cancers. There was no correlation between CD44v8–10 immunoreactivity and serosal, lymphatic, or lymph node invasion. However, there was significant correlation with CD44v8–10 immunoreactivity and venous invasion. CD44v8–10-positive cancers were more frequently associated with hematogenous metastasis than those which were immunonegative. There was an inverse association between CD44v8–10 immunoreactivity and peritoneal dissemination, especially in diffuse type adenocarcinomas. These observations indicate that CD44v8–10 may play a role in the metastasis of gastric cancer.**

Key words: Gastric cancer — CD44 variant exons 8–10 — Monoclonal antibody 44-1V — Hematogenous metastasis — Peritoneal dissemination

Hematogenous metastasis, lymph node metastasis and peritoneal dissemination are major prognostic factors for patients with gastric cancer. Therefore, it is essential to improve our understanding of the molecular basis of tumor metastasis and to have means for effective assessment of the metastatic potential of tumors. Human CD44 is thought to be a cell adhesion molecule and has been proposed to function in extracellular matrix binding, cell migration and lymphocyte homing.[1–6] A number of different isoforms of CD44 have been isolated and shown by amino acid sequence analysis to be generated by alternative mRNA splicing.[7,8] CD44 isoform carries 10 exons encoding a total of 338 amino acids in the membrane proximal extracellular region of the standard CD44. Various splice variants have been detected in a variety of human tumor cell lines and tumor tissues, such as breast cancer, lung cancer, stomach cancer, and colorectal cancer.[9–15] A previous study showed that CD44 isoform expression regulated tumor progression and metastasis. CD44 variant proteins containing sequences encoded by variant exons 5, 6 or 8–10 are related to tumor progression in colorectal cancer.[14]

We have established a murine monoclonal antibody (mAb), 44-1V, reactive with an epitope in CD44 variant exon 9 product using a fusion protein of CD44 containing variant exons 8–10. In this study, we analyzed the expression of CD44 variant exons 8–10 (CD44v8–10) protein in gastric cancer immunohistochemically using mAb CD44v8–10, and studied the correlation between CD44v8–10 immunoreactivity and clinicopathological findings.

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## MATERIALS AND METHODS

**Cell lines** Six human stomach cell lines were used. Human stomach cancer cell lines, MKN-1,and SCH, were obtained from the Japanese Cancer Research Resources Bank. The cell lines were cultured in Dulbecco's modified Eagle's medium containing 7.5% fetal bovine serum and cultured in a $CO_2$ incubator at 37°C.

**Tissue specimens** Gastric carcinoma lesions from 194 patients were investigated in this study. Both normal tissues and tumor samples were resected from each patient in the First Department of Surgery, Fukui Medical School, Fukui. All of the patients underwent gastrectomy combined with lymph node dissection. The cancers were reviewed according to the general rules of clinical and pathological studies on gastric cancer for histological type, serosal invasion, lymphatic invasion and venous invasion (Japanese Research Society for Gastric Cancer[16]). Lymph node metastasis was positive in 157 (80.9%) of the patients, hematogenous metastasis was positive in 50 (25.8%), and peritoneal dissemination was positive in 65 (33.5%).

**Western blotting** Cells were lysed in 0.01 *M* Tris buffer, pH 7.3, containing 0.15 *M* NaCl, 0.01 *M* $MgCl_2$, 0.5% Nonidet P-40, 1 m*M* phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO) and 20 U/ml of aprotinin (Bayer, Leverkusen, Germany). Nonidet P-40 lysates containing 100 $\mu$g of protein separated by sodium

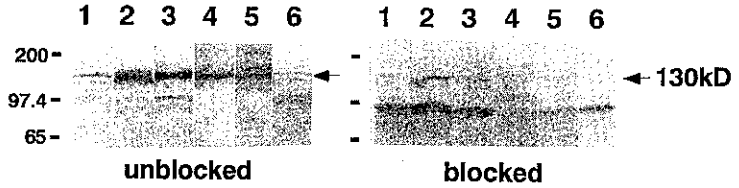


Fig. 1. Western blot analysis of human stomach cancer cell lines. A specific single band of 130 kD was recognized. This band disappeared or decreased after blocking with the fusion protein. 1, KATOIII; 2, MKN-1; 3, MKN-45; 4, NUGC-1; 5, NUGC-4; 6, SCH.

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gels were electrophoretically transferred onto polyvinylidenefluoride membranes (Immobilon, 0.22 $\mu$m pore size) (Nihon Millipore Kogyo K.K., Tokyo) for 4.5 h at 70 V in blotting buffer consisting of 0.025 *M* Tris, 0.192 *M* glycine and 20% methanol. The protein blots were incubated in phosphate-buffered saline (PBS) with 5% non-fat dried milk (Yukijirushi, Sapporo) and 0.02% $NaN_3$ at 4°C overnight. The membranes were incubated with mAb 44-1V at room temperature for 1 h, then washed with T-PBS (PBS containing 0.05% Tween-20) 3 times. Proteins were visualized using a Konica immunostaining HRP kit (Konica, Tokyo).

**mAb and immunohistochemical analysis** We have established a murine mAb reactive with an epitope in CD44 variant exon 9 product, and demonstrated that CD44v8–10 of 130 kD was specifically expressed in gastric cancer. The expression of CD44v8–10 in human stomach cell line MKN-1 was examined by immunohistochemistry. Frozen sections were fixed in acetone for 5 min. After preincubation with normal goat serum for 20 min, the sections were stained with mAb 44-1V (1 $\mu$g/ml) or with antibody which had been pre-adsorbed with 10 $\mu$g/ml of fusion protein.

Sections from the primary tumor and normal mucosa of each patient were stained with mAb 44-1V. Specimens were fixed in 10% formalin, embedded in paraffin and sectioned at a thickness of 4 $\mu$m. Sections were dewaxed and endogenous peroxidase activity was blocked by incubation for 30 min in 1% hydrogen peroxidase in methanol. The hydrated sections were incubated in normal goat serum at room temperature for 20 min to reduce nonspecific staining, and incubated overnight at 4°C with mAb 44-1V (1 $\mu$g/ml). The slides were washed with Tris-buffered saline (TBS), and incubated with biotinylated goat anti-mouse immunoglobulin (DAKO Patts, Copenhagen, Denmark) at room temperature for 20 min, then with a 1:100 dilution of streptavidin-biotin-peroxidase complex (DAKO Patts) at room temperature for 20 min. The peroxidase activity was visualized with 3,3′-diaminobenzine tetrahydrochloride and $H_2O_2$ in 0.05 m*M* Tris buffer, pH 7.2. Finally, the slides were lightly counterstained with hematoxylin. Negative control staining was performed in the absence of the primary antibody to CD44v8–10. Intensity of tumor staining with mAb 44-1V was classified as follows: negative, staining less than 25% of the cancer cells; positive, more than 25%.

**Statistical analysis** Statistical analyses of data were performed using the chi-squared test. The outcomes from different groups of patients were compared by means of the generalized Wilcoxon's test. Differences were considered significant when *P* values were less than 0.05.

## RESULTS

**Western blot analysis** The expression of CD44v8–10 protein in stomach cell lines was analyzed by immunoblotting using the specific mAb 44-1V. A representative blot is shown in Fig. 1. Specific bands of 130 kD were observed in stomach cancer cell lines. In several stomach cell lines, MKN-1, MKN-45, NUGC-1, and NUGC-4, CD44v8–10 protein was expressed strongly. These bands that disappeared or decreased after blocking with the fusion protein were significantly reactive.

**Immunohistochemical staining** Strong immunoreactivity was localized on the cell membrane of MKN-1 (Fig. 2a). The specificity of the immunohistochemical staining was confirmed in a serial section stained with mAb 44-1V that had been pre-adsorbed with the fusion protein (Fig. 2b). Under these conditions, the staining of tumor cells was entirely abolished.

Of the 194 specimens of gastric carcinoma, 65 (33.5%) reacted positively with mAb 44-1V. CD44v8–10-positive immunoreactivity was seen on the cell membranes of gastric carcinoma cells, and a few cells also had weakly stained cytoplasm (Fig. 3), while adjacent normal gastric mucosa, except the crypt base, showed no staining by mAb 44-1V. The correlations between CD44v8–10 expression and clinicopathological parameters are shown in Table I. With regard to histological type, 41.8% (38 of 91) of differentiated adenocarcinomas and 26.2% (27 of 103) of poorly differentiated or signet ring cell carcinomas were stained positively for CD44v8–10 protein. A significant correlation was observed between CD44v8–10 protein expression and histological type ($P<0.05$). However, there was no significant correlation between CD44v8–10 immunoreactivity and serosal invasion, lymphatic invasion, or lymph node metastasis. CD44v8–10

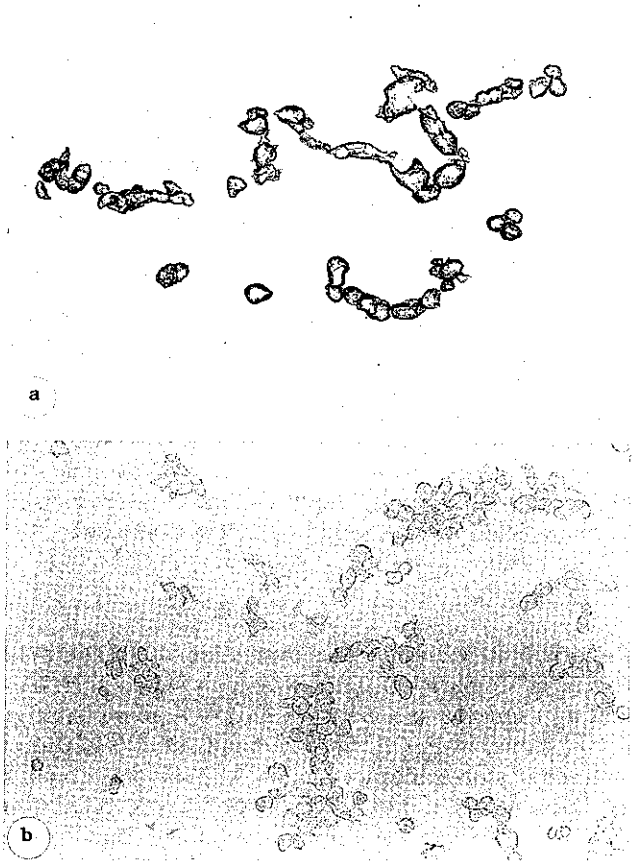


Fig. 2. Immunostaining of CD44 isoform containing variant exons 8–10 product in human stomach cancer cell line MKN-1. CD44v8–10-positive immunoreactivity was seen on the cell membranes of MKN-1 (a). Stomach cancer cell line MKN-1 showed no staining by mAb 44-1Y which had been pre-adsorbed with 10 $\mu$g/ml of the fusion protein (b).

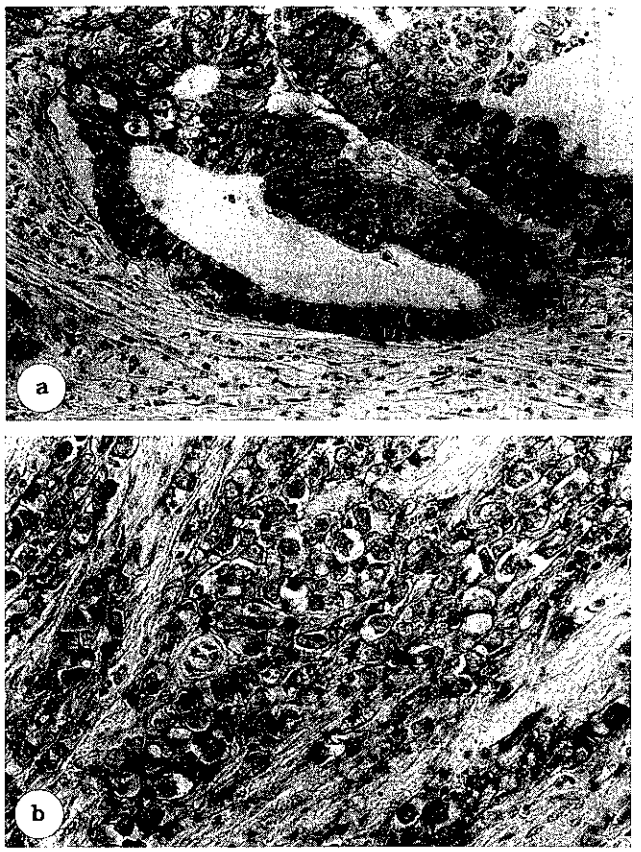


Fig. 3. Immunostaining of CD44 isoform containing variant exons 8–10 product in gastric carcinoma. The staining was intense, mainly on the cell membranes. a, well differentiated adenocarcinoma; b, poorly differentiated adenocarcinoma.

Table I. Correlation between CD44v Expression and Clinicopathological Findings

| | Cases | CD44v positive | |
|---|---|---|---|
| Histological type | | | |
| differentiated adenocarcinomas | 91 | 38 (41.8%) | $P=0.022$ |
| poorly differentiated adenocarcinomas and signet ring cell carcinomas | 103 | 27 (26.2%) | |
| Serosal invasion | | | |
| negative | 87 | 29 (33.3%) | $P=0.964$ |
| positive | 107 | 36 (33.6%) | |
| Lymphatic invasion | | | |
| negative | 24 | 8 (33.3%) | $P=0.985$ |
| positive | 170 | 57 (33.5%) | |
| Venous invasion | | | |
| negative | 71 | 17 (23.9%) | $P=0.032$ |
| positive | 123 | 48 (39.0%) | |
| Lymph node metastasis | | | |
| negative | 37 | 12 (32.4%) | $P=0.878$ |
| positive | 157 | 53 (33.8%) | |

immunostaining was observed in 39.0% of 123 patients with and 23.9% of 71 patients without venous invasion (Table I). The CD44v8–10-positive rate was significantly different between the two groups ($P<0.05$). There was no significant correlation between CD44v8–10 immunoreactivity and stage (Table II). The CD44v8–10-positive rate was 26.4% for 144 tumors without and 54.0% for 50 tumors with hematogenous metastasis; the difference between these two groups was significant ($P<0.01$). In differentiated adenocarcinoma, hematogenous metastasis is more often seen in cases with CD44v8–10-positive tumors than in those with CD44v8–10-negative tumors (Table III). CD44v8–10 immunoreactivity was observed in 38.0% of 129 patients without and 24.6% of 65 patients with peritoneal dissemination (Table IV). There was an inverse association between CD44v8–10 expression in gastric cancer and peritoneal dissemination. In diffuse type adenocarcinoma especially, peritoneal metastasis was present more frequently in CD44v8–10-negative tumors than in positive tumors ($P<0.05$). Five-year survival was achieved in 38.4% of the 129 patients with CD44v8–10-negative tumors and in 40.3% of the 65 patients with CD44v8–10-positive tumors. There was no significant correlation between CD44v8–10 and prognosis in these cases.

Table II. Correlation between CD44v Expression and Stage

| Stage | Cases | CD44v positive |
|---|---|---|
| I | 22 | 7 (31.8%) |
| II | 21 | 6 (28.6%) |
| III | 50 | 20 (40.0%) |
| IV | 101 | 32 (31.7%) |

$P=0.716$

## DISCUSSION

We examined the expression of CD44v8–10 protein by western blot analysis and immunohistochemistry in gastric cancers using mAb 44-1V. This antibody is a mAb to CD44v8–10 glycoprotein which contains the variant exons 8–10 products. On western blots, a single band with a molecular weight of 130 kD was recognized in several stomach cancer cell lines. CD44v8–10 immunoreactivity was detected mainly on the cell membranes of gastric cancer cells, and a few cells had weakly stained cytoplasm. Adjacent normal gastric mucosa, with the exception of the crypt base, showed no staining by mAb 44-1V. Heider *et al.*[10] showed that intestinal-type gastric carcinomas were strongly positive for epitopes encoded by variant exons 5 and 6, whereas diffuse-type adenocar-

Table III. Correlation between CD44v Expression and Hematogenous Metastasis

| | Cases | CD44v positive | |
|---|---|---|---|
| Liver metastasis | | | |
| negative | 166 | 50 (30.1%) | $P=0.015$ |
| positive | 28 | 15 (53.6%) | |
| Hematogenous metastasis | | | |
| All cases | | | |
| negative | 144 | 38 (26.4%) | $P<0.01$ |
| positive | 50 | 27 (54.0%) | |
| Differentiated type | | | |
| negative | 59 | 17 (28.8%) | $P<0.01$ |
| positive | 32 | 21 (65.6%) | |

These data were at the first operation except for hematogenous metastasis, which included those that appeared during observation.

Table IV. Correlation between CD44v Expression and Peritoneal Metastasis

| | Cases | CD44v positive | |
|---|---|---|---|
| Peritoneal metastasis | | | |
| All cases | | | |
| negative | 129 | 49 (38.0%) | $P=0.062$ |
| positive | 65 | 16 (24.6%) | |
| Diffuse type | | | |
| negative | 58 | 20 (34.5%) | $P=0.030$ |
| positive | 45 | 7 (15.5%) | |

cinomas predominantly expressed only variant exon 5. In addition, they reported that established variant CD44-specific antibodies against variant exons v8–10 did not react in either type of gastric carcinoma. In contrast, Mayer *et al.*[11] found a good correlation between the expression of total CD44 and that of variant exon 9-containing isoforms using an antibody directed against CD44 variant exon 9, and showed that CD44 isoforms containing variant exon 9 are present in gastric carcinomas. We also showed that 65 (33.5%) of 194 gastric cancer specimens were positive for epitopes encoded by variant exons v8–10. With regard to histological type, the expression of CD44 glycoprotein with variant exons v8–10 was significantly higher in well differentiated adenocarcinomas than in poorly differentiated adenocarcinomas or signet ring cell carcinomas.

The functions of CD44 variant isoforms are largely unknown, although it is thought that CD44 variants may play important roles in tumor growth and metastasis.[17–20] A highly metastatic variant of a rat pancreatic carcinoma displayed a specific CD44 isoform not expressed by the poorly metastatic variant, and it is this form which is responsible for the enhanced metastatic potential.[17] In addition, several reports have shown a correlation between metastatic potential and expression of CD44 variants in various human tumors.[9, 11–15] CD44 variant isoforms may play an important role in tumor growth and metastasis in colorectal cancer patients. Tanabe *et al.*[13] reported that overexpression of CD44 variants may increase the metastatic potential of cancers. Our previous northern blot analysis indicated that expression of the CD44 splice variant containing exons v8–10 is related to hematogenous metastatic potential.[15] In gastric cancer, isoforms of variant CD44 containing exon v9 were found to be associated with distant metastases, and were significantly and positively correlated with tumor recurrence and mortality.[11] In this study, we demonstrated that there was a significant correlation between CD44v8–10 immunoreactivity and venous invasion, and found that the rate of CD44v8–10 expression was significantly higher in tumors from patients with liver metastasis than in those without. However, there was no correlation between CD44v8–10 immunoreactivity and prognosis. A decrease in CD44v8–10 expression was noted in tumors associated with peritoneal metastasis when compared to those without. Our findings suggest that CD44v8–10 may be involved in hematogenous metastasis, and may play a role in the suppression of peritoneal metastasis of gastric cancer.

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